HIGH THROUGHPUT SCREENING OF FLAX (*Linum usitatissimum* L.) CYCLOLINOPEPTIDES

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By
CLARA MARISA OLIVIA

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ABSTRACT

Flax (*Linum usitatissimum* L.) seed is a rich source of potentially valuable cyclic peptides (CLs). The current research investigated the genotypic and environmental variation of CLs expressed in the Flax World Core Collection by means of high-throughput extraction and rapid HPLC analysis. The collection is a representative subsample of the widest diversity of flax gene resources. The performance of monolithic and microparticulate HPLC columns were compared for their efficiency in rapid reversed phase liquid chromatographic separation of CLs. CL analysis time was reduced to 1.5 min at a flow rate of 3.0 $mL \cdot min^{-1}$ using a monolithic Chromolith® SpeedROD. The column performed over 2000 injections without measurable changes in performance under these conditions. The optimum extraction conditions of CLs were also established. Total CL content was proportional to sum of UV absorbance (mAU) of the observed peptide chromatographic peaks at 214 nm. The total mAU of samples extracts from core collection samples planted in Saskatoon ranged from 71.4 to 223.7 mAU with a mean value of 153.5 ± 24.3 mAU ($\bar{x} \pm$ SD), whereas the total mAU from samples grown at the Morden site ranged from 96.2 to 243.6 mAU with a mean value of 171.3 ± 24.0 mAU. The highest total CL content was observed in cultivar 'AC Watson', whereas, 'Primus' and an unnamed accession (CN 101580, TMP 10121) exhibited the highest ratio CLs expressed by NCBI gene sequence AFSQ01016651.1 to peptides expressed by gene sequence AFSQ01025165.1. In contrast, the lowest total CL content and ratio of these gene products was observed in 'Hollandia' and 'Z 11637'. No correlation between CL content and plant height, stem branching, petal colour, seed colour, seed weight, seed oil content, or α-linolenic content was evident. In addition to the known peptides, novel peptides 1-Met-CLN, 1-Mso-CLN, and 1-Msn-CLN were identified from 'Hollandia' and 'Z 11637'. The variation of CL content and composition of flaxseed from the core collection reveals that genetics may benefit flax breeding programs by allowing the selection of flax accessions with improved composition and functional properties.

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LIST OF ABBREVIATIONS

ALA	α-linolenic acid
CDC	Crop Development Centre
CHR	Chromolith® High Resolution (MerckKGaA Darmstadt, Germany)
CL	Cyclolinopeptide
CN	Canadian National Accession Number
CP	Chromolith® Performance (MerckKGaA Darmstadt, Germany)
CsA	Cyclosporine A
CSR	Chromolith® SpeedROD (MerckKGaA Darmstadt, Germany)
CV	Coefficient of variation
DAD	Diode array detector
DMSO	Dimethylsulfoxide
DUS	Distinctness, Uniformity, Stability
FTIR	Fourier transform infrared spectroscopy
HR-FABMS	High resolution fast atom bombardment mass spectrometry
PGRC	Plant Gene Resources of Canada
POR	POROS R1/20 (Applied Biosystems)
HPLC	High performance liquid chromatography
HPLC/ESI-MS	High performance liquid chromatography/electrospray ionization – mass spectrometry
HPLC/ESI-MS/MS	High performance liquid chromatography/ electrospray ionization tandem mass spectrometry
NMR	Nuclear magnetic resonance
SD	Standard deviation
SDG	Secoisolariciresinol diglucoside
TMP	Temporary Numbers
UPLC	Ultra performance liquid chromatography
ZEX	ZORBAX Eclipse XDB-C_{18} (Agilent)

CHAPTER 1
INTRODUCTION

Domestication of flax or linseed (*Linum usitatissimum* L.) for the purpose of fibre and oil dates back to 10,000 BC (Zohary and Hopf, 2000). The annual herbaceous plant was proposed to have originated in the Middle East although secondary diversity centers were identified in the Ethiopia, India, Central Asia, and Mediterranean basin (Vavilovl, 1926; Zohary and Hopf, 2000). The average worldwide production of linseed between 1999 and 2010 was 2,100,000 tonnes, with major producers including Canada, China, USA, Ethiopia, India, and a few European countries (FAO, 2012). During this period, there was an average of 624,000 ha of linseed grown in Canada with an average annual yield of 1,219 kg/ha (FAO, 2012). According to Statistics Canada (2000), Canada contributes about 36% of the total worldwide linseed production and exports about 90% to Europe, USA, and Japan. The majority of Canadian flaxseed is produced in the prairie provinces (Alberta, Manitoba, and Saskatchewan) (Statistics Canada, 2000), with Saskatchewan contributing more than two thirds of the total production in western Canada (Flax Council of Canada, 2000).

An increased recognition of the need for healthy lifestyle has boosted the consumption of foods that offer health benefits. Flax is sold commercially in several products including flax oil, flax oil capsules and flaxseed (ground and whole). The oil may be consumed directly or added to foods like salads, while the seed is often incorporated in foods such as yoghurt, muffins, and beverages. As a functional food, flax offers a high concentration of the essential omega-3 fatty acid α-linolenic acid, which is reported to prevent cancer and reduce the risks of chronic diseases, such as coronary artery disease, atherosclerosis, and type II diabetes (Cunnane *et al*., 1993). Flaxseed has better palatability, shelf life and a wider range of nutritional components than flax oil products. Specifically, flaxseed products provide dietary fibre, lignan, and protein that are not available in flax oil, whereas flax oil is an excellent source of linolenic acid and cyclolinopeptides (CLs).

The cardioprotective effects of flaxseed, associated with the reduction of LDL and total cholesterol after consumption of either raw or defatted flaxseed (30 and 50 g/day) have been reported in several clinical studies (Cunnane *et al*., 1993; Clark *et al*., 1995; Jenkins *et al*., 1999). Consumption of flaxseed by patients suffering lupus nephritis also conferred benefits in terms of improved renal function and decreased progression of atherogenic and inflammatory processes (Jenkins *et al*., 1999), without compromising antioxidant status (Cunnane *et al*., 1995). Flaxseed (38 g/day) taken for six weeks reduces the rate of bone resorption in post-menopausal women (Arjmandi *et al*., 1998). Its phytoestrogenic and therapeutic activity have been proposed to exert a beneficial effect in reducing the risk of hormone-related cancers (Lampe *et al*., 1994; Arjmandi *et al*., 1998; Kurzer *et al*., 1998; Haggans *et al*., 1999; Nesbitt *et al*., 1999).

The Flax World Core Collection comprises 380 accessions, selected by Plant Genetic Resources of Canada to represent the widest genetic variation of flax. Knowledge of the chemical composition of flax varieties is crucially important in order to understand genetic variation and potentially develop value-added uses for flax. The presence of CLs in flaxseed may contribute to some functional benefits of flaxseed. Numerous studies have shown that CLs are biologically active. In *in-vitro* studies, CLA inhibits activation and proliferation of T-lymphocyte cells (Wieczorek, *et al.*, 1991), protects liver from specific poisonous agents (Kessler *et al.*, 1986), and suppresses immunity (Wieczorek, *et al.*, 1991; Górski *et al.*, 2001). Peptides 1-Met-CLB, 1-Msn-CLB, and 1-Mso-CLE exhibit immunosuppressive activities (Morita *et al.*, 1999; Morita and Takeya, 2010). These peptides have potential applications as therapeutic agents for suppressing hemolytic anemia and post adjuvant arthritis, postponing skin allograph rejection, and delaying hypersensitivity response (Gaymes *et al*., 1997; Siemion *et al*., 1999). This thesis focused on determining the type and content of CLs in flaxseed accessions from the core collection by means of high throughput screening. The objectives of this study are as follow:

1. to develop methods for detection of CLs using monolithic and microparticulate reversed phase HPLC columns;
2. to establish a high throughput extraction method for screening flax CLs and subsequent application of the established method to determine profile of CLs from flaxseed accessions in the World Core Collection, grown in Saskatoon, SK and Morden, MB in 2009; and

3. to confirm and partially characterize novel CLs found in *L. usitatissimum* L. accession ‘Hollandia’ and ‘Z 11637’.

CHAPTER 2
LITERATURE REVIEW

2.1 *Linum usitatissimum* L.

Flax belongs to the family Linaceae and genus *Linum* (Linnaeus, 1857). The species name *usitatissimum* meaning "most useful" best describes the various applications of flax (McHughen, 1992). A variety of flaxseed products are available including flax oil, whole and milled flaxseed. The oil may be consumed directly or added to foods like salads, while the seed is often incorporated in foods such as muffins, bagels, and multigrain breads. High concentrations of the essential omega-3 fatty acid α-linolenic acid (ALA) are the primary basis of flaxseed popularity within both the food and feed market. The known health benefits of ALA (Kamano *et al.*, 1989; Fritsche and Johnston, 1990; Cunnane *et al.*, 1995; Caughey, 1996; Arjmandi *et al.*, 1998; Bemelmans, 2000; Lemay *et al.*, 2002; Djousśe *et al.*, 2003a; b; 2005; 2006; Takeuchi *et al.*, 2007; Tao *et al.*, 2008) have prompted supplementation of animal feeds with flaxseed products as a means to increase the omega-3 fatty acid content in the animal tissues and products. The presence of ALA, as well as other health-promoting compounds, such as protein, lignan, and dietary fibre in flaxseed has established the role of flaxseed as functional food. On the other hand, the stem of flax has been widely known for its bast fibre. Flax fibre is described as lustrous, soft, and flexible (Singh *et al.*, 2001). Flax fibre is a raw material in textile industry for linen cloth and ropes, and also in paper industry for specialty papers including cigarette paper and currency notes (Van Dam *et al.*, 1994). Flax fibre is also used to make mats (Berglund, 2002) and low-cost roofing tiles. The rapid drying characteristic of flax oil upon exposure to the air, as well as its low environmental footprint makes flax oil a superior choice for paint, resin, soap, ink, flooring (linoleum), and varnish industries.

2.2 General description of the plant

Selective breeding of *L. usitatissimum* has led to development of varieties that are specialized as either oilseed or fibre types. The fibre and oilseed types may be identified by their phenotypic characteristics. Accessions grown primarily for oilseed production are generally shorter in height, highly branched and possess more seed bolls compared to the relatives cultivated for fibre production (Jhala and Hall, 2010). Linseed and fibre flax perform best in different regions. Linseed is mainly grown in climates with low moisture and more sunlight, which is characteristic of highland and subtropical regions (Cullis, 2007). Northern temperate regions with high air humidity and low temperature are ideal locations for fibre flax cultivation, indicating less drought tolerance of fibre flax (Bunting, 1951).

Mature plants of cultivated flax grow to heights between 40 to 91 cm, depending on variety, soil fertility, available moisture, and seeding density (Flax Council of Canada, 2002). The time from seeding to harvest varies between 90 to 125 days. The flax life cycle consists of vegetative, flowering, and maturation stages. High temperature, water stress, and diseases can shorten any of these growth stages (Flax Council of Canada, 2002). Flax tolerates a wide range of climates but grows best in moderate to cool conditions (Casa *et al.*, 1999). High temperatures during the ripening phase decreases the seed weight, the number of seeds per capsule, and reduces oil yield and quality (Dean Dybing and Zimmerman, 1965). It also appears that flax plants yield better on well-drained, medium to heavy textured soils, especially clay or silty loams of about pH 6 (Hocking *et al.*, 1987). In Canada, flax is adapted to brown-black chernozemic soil of the Prairies stretching from longitude 96º to 121º West, from latitude 49º to 59º North and from 225-1000 m above sea level (Dorell, 1975). Levels of nutrients in the soil vary greatly among regions, with soil types, fertilizer use, and cropping history (Flax Council of Canada, 2002).

The fibre flax plant has a short, branched taproot with a leading shoot usually measuring more than 1 m and lateral branches extending approximately 30 cm (Crooks, 1933). The flax stem is of special interest as it contains fibre. The quality of flax fibre is determined, in part, by the degree of stem branching (Diederichsen and Richards, 2003). A long primary fibre, used in the textile industry can only be obtained from the technical stem, defined as the length of stem without side branches (Kulpa and Danert, 1962). Fibre flax is usually planted at high density so that each plant develops only one main shoot, with fewer side branches, and hence, less seed

production (Diederichsen and Richards, 2003). Cross-sections of flax stem are shown in Figure 2.1 (Elladi, 1940). The fibre is located in the pericambial part of the stem, between the cortex and the phloem regions (Figure 2.1).

When given sufficient opportunity to mature all branches of flax shoot terminate in flowers. Flax blooms in a panicle-like arrangement (Diederichsen and Richards, 2003). Each flower only blooms for several hours, opening early in the day and shedding most of the petals by noon (Cullis, 2007). The flower parts (sepals, petals, carpels, anthers) occur in units of five. The corolla shapes into a tube, funnel or bowl, with a diameter of 10-32 mm (Kulpa and Danert, 1962). Flax varieties can be distinguished by the colour of petals, filaments, and the anthers. The most frequent colour of petals is blue, followed by white, although light blue, violet, red violet, and pink are possible (Tammes, 1928; Dillman and Brinsmade, 1938). The mature fruit of a flax plant is round with capsule-like shape, alternatively called boll measured approximately 6-9 mm wide (Barnes *et al.*, 1960). The boll consists of five segments separated by a wall (septum), with two seeds in each segment (Dillman and Brinsmade, 1932). Therefore, each capsule has a maximum of ten seeds, though less seeds per boll are normal (Cullis, 2007). Ripening of the fruit occurs 20 to 25 days after flowering depending on the genotype. The ripe bolls are either open slightly (fibre flax) or completely closed (oilseed flax) (Dillman and Brinsmade, 1932).

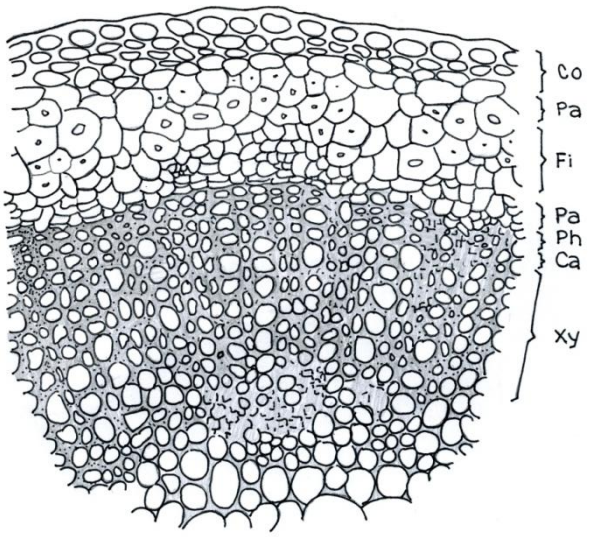


Figure 2.1 Cross-section of flax stem. Co: cortex; Pa: parenchyma, Fi: fibre fascicle; Ph: phloem; Ca: cambium; Xy: secondary xylem (Adapted from Elladi, 1940)

2.3 General structure of flaxseed

The seed of flax can be described as flat and oblong elliptic with rounded base and a pointed tip (Diederichsen and Richards, 2003). Flaxseed ranges in colour from light yellow, olive to deep brown and measures about 4-6 mm, depending on variety and growing conditions (Daun *et al.*, 2003). The seed is composed of a smooth, often shiny seed coat (hull) covering an embryo consisting of two cotyledons, the hypocotyl and the radicle surrounded by a thin endosperm layer (Peterson, 1958). The weight of seed is approximately 5 ± 1 mg with positive correlation between seed weight and oil content (Geddes and Lehberg, 1936). The cotyledons, embryo and seed coat and endosperm (hull) constitute 55, 4, and 36% of the seed mass, respectively (Daun *et al.*, 2003). Cross-sections of flaxseed are provided in Figure 2.2 (Gassner, 1951). The seed coat or true hull of mature flaxseed consists of five layers (Hayward, 1938; Boesewinkel, 1980; Daun *et al.*, 2003). From the periphery to the inside, there are the epidermis (mucilage cells), ring cells (or round cells), sclerenchymatous layer (or fibre cells), transversal cells (or cross cells), and pigment layer (Daun *et al.*, 2003).

The outermost layer of the seed coat consists of cells that appear rectangular (longitudinal section) and polygonal when viewed from the surface (Hayward, 1938). The plant cuticula is made up of predominantly lipid material that form continuous thin layer on the outer wall of epidermal cells (Kirkwood, 1999). The mucilaginous carbohydrate material deposited within the extracellular space of the epidermal cells below the cuticula contributes to the shiny appearance of mature flaxseed (Flax Council of Canada, 1992; Tarpila *et al.*, 2005). The production of seed mucilage, known as myxospermy has been observed in families such as Plantaginaceae, Brassicaceae, Acanthaceae and Linaceae (Grubert, 1974; 1981; Fahn, 1979; Ryding, 2001; Kreitschitz, 2009). Mucilage has been proposed to play vital roles in successful germination (mediation of seed dispersal through attachment to animal vectors or soil, regulation of seed oxygen concentration, and facilitation of seed hydration) (Grubert, 1974; Fahn, 1982; Ryding, 2001; Kreitschitz, 2009).

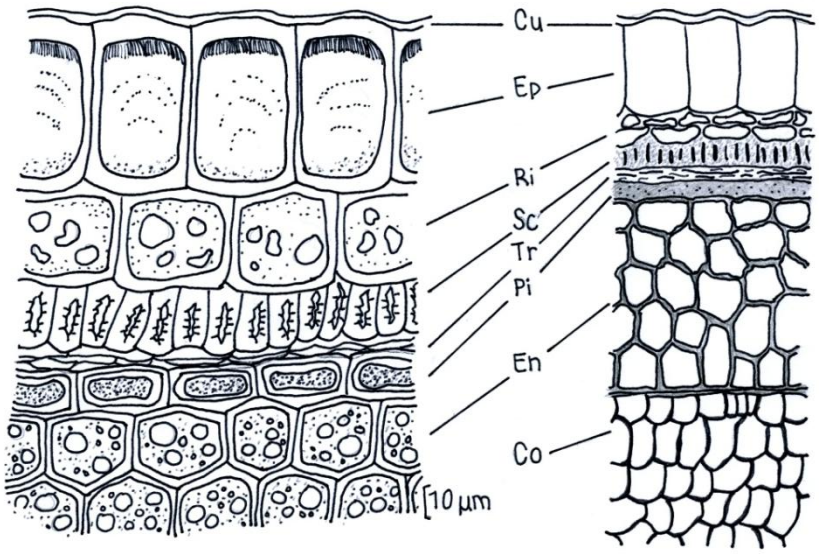


Figure 2.2 Cross-section of flaxseed. Cu: cuticula; Ep: epidermis, Ri: ring-cells; Sc: sclerenchyma; Tr: transversal-cells; Pi: pigment-cells; En: endosperm; Co: cotyledon (Adapted from Gassner, 1951)

The ring cells appear more or less round when seen from the surface (Rüdiger, 1942; 1954). They are parenchymatous in origin (Freeman, 1995) and have considerable intercellular space (Hayward, 1938). The outer appearance of the seed colour can be affected by the ring cells when a dark, tannin-like substances and occasionally chlorophyll is present in the intercellular space (Diederichsen and Richards, 2003). The third structural layer of the seed coat constitutes sclerenchyma fibre. Fibre cells are longitudinally oriented, porous, thick-walled, and lignified (Hayward, 1938; Boesewinkel, 1980; Freeman, 1995). The layer is mostly colourless, but dark yellow appearance has been reported (Diederichsen and Richards, 2003). Rüdiger (1942) reported that genotypic variation in the thickness of sclerenchymatous layer of cultivated *Linum*, although this layer is generally thicker in wild *Linum* compared to the cultivated flax (Diederichsen and Richards, 2003). The layer below the fibre cells is composed of transversal cells or cross cells (Diederichsen and Richards, 2003; Freeman, 1995). The transversal cells are colourless, thin-walled, highly compressed in nature (Hayward, 1938) and oriented irregularly (Diederichsen and Richards, 2003). The innermost layer contains the pigment cells (Boesewinkel, 1980), which are polygonal and square shaped when viewed in a pericline section (Diederichsen and Richards, 2003). The anatomical layer is reported to be single-layered with thick porous walls (Hayward, 1938). Boesewinkel (1980) observed that tannin pigments of yellow-brown colour deposited in the cavities of the pigment cells are responsible for the testa colour. It was reported that in yellow-seeded flax, these cells are often absent or, in some instances, are present but do not contain pigment (Freeman, 1995).

2.4 The Flax World Core Collection

The great adaptability and diversity of flax has obscured the origin of cultivated flax (Lay and Dybing, 1989), as well as the initial purpose of flax cultivation. Plant breeding has further narrowed the genetic variation in flax (Tanksley and McCouch, 1997). Diederichsen and Fu (2006) have demonstrated low genetic diversity in cultivated flax during screening of DUS (distinctness, uniformity, and stability) criteria using morphological descriptors. Further decrease of genetic variability may occur with the cultivation of only modern accessions in future breeding (Vromans, 2006). Knowledge about the genetic distribution within flax varieties is crucially important in order to discover further uses of flax (Tanksley and McCouch, 1997). The total number of flax germplasm accessions preserved in national genebanks, international research and

other public genebank around the world is estimated at about 48,000 (Diederichsen, 2007). Plant Gene Resources of Canada (PGRC) has assembled more than 3,000 flax lines from 76 countries over the last 40 years. The collection was generated through exchanges with flax breeders and genebanks in the United States, Poland, Chile, Turkey, Czech Republic, Russia, Germany, etc. Due to genetic similarity of these varieties, PGRC has selected 380 accessions, dubbed as World Core Collection that embodies the diversity found in the entire collection. The morphological traits and oil composition of each accession are accessible through PGRC website (http://pgrc3.agr.gc.ca/acc/search-recherche_e.html) (Table 2.1, Figure 2.3).

The genetic integrity and stability of flax germplasm must be preserved with due diligence during seed handling and storage. Cultivated flax is predominantly self-pollinating. The frequency of cross-pollination may reach 1.47% (Robinson, 1937). However, more recent studies reported out-crossing rates as high as 6%, which may be attributed to both genotypic and environmental influences (McGregor, 1976; Free, 1993). Wind-induced cross-pollination is rare due to the mass of flax pollen grain (Diederichsen and Richards, 2003). Nevertheless, considerable isolation distances between the different genotypes of flax and reduction of pollinating insects (Abrol and Kotwal, 1996) are recommended to avoid cross-pollination. Seeding, harvesting, or seed cleaning might also create problems for regeneration of the accessions. Physical mixture of genotype might be undistinguishable except for existing characterization data to detect any deviations.

Table 2.1 Variation found in the flax core collection (Plant Gene Resources of Canada, 2010)

Traits	**N**	**Range**	**Mean**	**SD**
Length Of Flowering Days	305	9 - 60	29	7
Height (cm)	368	17 - 125	59	18
Total seed oil (% dry weight)	352	33.8 - 45.7	38.3	2.1
α-linolenic acid [§]	355	39.4 - 67.6	55.2	4.8
Linoleic acid [§]	352	7.3 - 27.4	13.5	2.7
Oleic acid [§]	352	11.6 - 31.8	20.2	3.6
Palmitic acid [§]	352	2.9 - 6.8	5.1	0.6
Stearic acid [§]	352	2.2 - 8.6	4.3	1.3

[§] Percentage in total seed oil

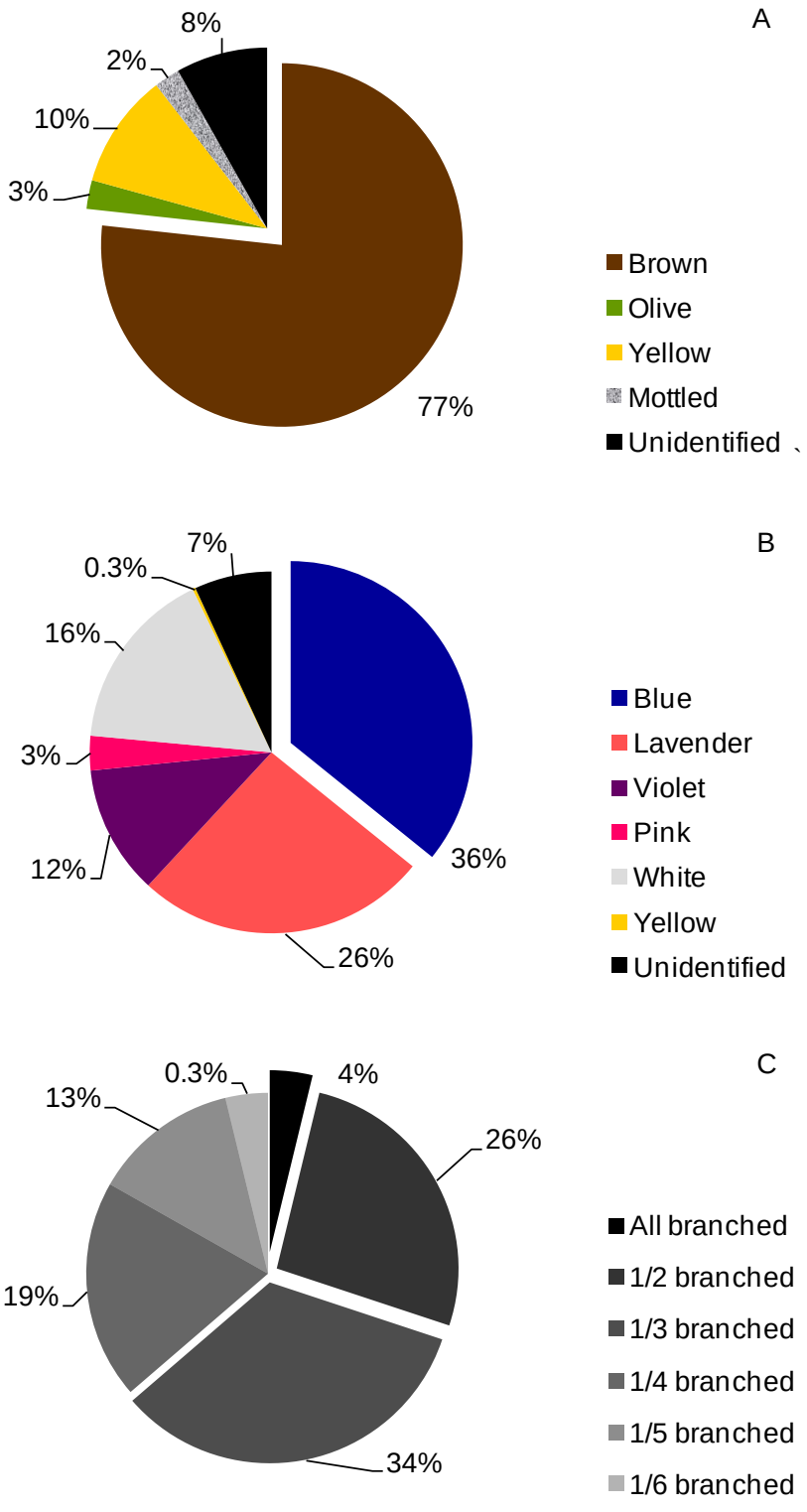


Figure 2.3 Variation of (A) seed colour, (B) petal colour, and (C) stem branching in the flax core collection (Plant Gene Resources of Canada, 2010)

2.5 Chemical constituents of flax

Flaxseed is rich in lipid, dietary fibre, and protein. Canadian Grain Commission (2001) reported the average composition of commercial brown Canadian flaxseed as 41% lipid, 28% dietary fibre, 20% protein, 7.7% moisture, and 3.4% ash. Minor components include lignan, phenolics, minerals, vitamins, cyanogenic glycosides, phytic acid, linatine, and cyclic peptides (Bhatty, 1995; Morita *et al.*, 1997, Matsumoto *et al.*, 2002). Compositions of flax vary considerably according to genotype, environment, and seed processing (Daun *et al.*, 2003). The method of analysis may also impact reported composition.

2.5.1 Lipid content

The oil content in flaxseed has been reported to range from 38% (Van Uden *et al.*, 1994; Oomah and Mazza, 1997) to 45.6% (Canadian Grain Commission, 2001). The oil content of flax varies mainly due to genotypic and environmental effects (Van Uden *et al.*, 1994; Oomah and Mazza, 1997), although seed processing and methods of analysis may also be a factor in reported variation (Daun *et al.*, 2003). Flaxseed oil is mainly present in the cotyledons (75%) while the endosperm and seed coat contain 22% of the oil (Dorrel, 1970). Oil in the cotyledons and endosperm are stored in the form of oleosomes, or cell bound microscopic oil droplets. Principal characteristic distinguishing *L. usitatissimum* L. from other oilseeds is its high concentration of polyunsaturated fatty acids (PUFAs). ALA (18:3n-3) constitutes about 52%, whereas, linoleic acid about 16% (18:2n-6) of the total fatty acids. Other fatty acids present in the oil include oleic (20%), palmitic (6%), and stearic acids (4%) (Green, 1990). Linoleic acid and ALA are essential fatty acids due to their important physiological and biochemical functions within the human body. ALA is an intermediate in producing long chain omega-3 PUFAs (EPA, 20:5n-3 and DHA, 22:6n-3), that are known to regulate immune function and inflammation in higher animals (Mantzioris *et al.*, 1994, 1995).

2.5.2 Protein content

Protein content in flaxseed ranges from 10.5 (Bajpai *et al.*, 1985) to 31% (Salunkhe and Desai, 1986). Differences in protein contents are largely contributed by both genetic and environmental factors (locations and seasons) (Oomah and Mazza, 1993). Approximately 30% of flax protein is stored in the seed coat and endosperm while about 56-70% is present in cotyledons

(Dev *et al.*, 1986; Sosulski and Bakal, 1969). As is common in other oilseeds, a negative correlation exists between seed protein and oil content (Naqvi *et al.*, 1987; Bajpai *et al.*, 1985). The interaction between flaxseed protein and mucilage may play a significant role in influencing blood glucose level and stimulating insulin secretion, thereby reducing glycaemic response (Nuttall *et al.*, 1984). Flaxseed protein may also reduce colon luminal ammonia, providing protection against ammonia production and consequent tumour promoting effects (Clinton, 1992). Flaxseed protein is high in branched-chain amino acids and low in aromatic amino acids, comparable with that of soy flour (Oomah, 2001). Flaxseed meal and soy flour also have a similar pattern of essential amino acids, indicating flaxseed meal has potential as a source of nutrient-rich plant protein (Oomah and Mazza, 1993). The high levels of His, Glu, and Arg in flaxseed protein have the potential to boost immune function (Oomah, 2001). Flaxseed protein contains high levels of methionine and cysteine, amino acids known to improve antioxidant levels, hence, the ability to stabilize DNA during cell division and reduce certain forms of colon cancer risk (Oomah, 2001).

2.5.3 Carbohydrate content

Flaxseed is an excellent source of soluble and insoluble dietary fibre, both accounting for about 28% of the seed weight (Daun *et al.*, 2003). The insoluble fibre consists of lignin, cellulose, and non-starch polysaccharides found within the plant cell walls. The soluble fibre in flax is more commonly called flax mucilage or gum, accounts for 6-8% dry weight of whole seed depending on the accession (Oomah *et al.*, 1995; Cui *et al.*, 1996). Mucilage is a water-soluble hydrocolloid, generally isolated through aqueous extraction of the epidermal layer of intact whole seed followed by ethanol precipitation (Cui *et al.*, 1994). Dietary fibre helps to increase satiety, control blood glucose, reduce blood lipid concentration, and promotes laxation. It acts a bulking agent in the gut by increasing the viscosity of digested material and thus increases stool weight, while decreasing material transit time through the gut. Hence, diets rich in dietary fibre can help reduce the risk of colourectal cancer, diabetes, obesity, heart disease, and inflammation (Brenna, 2005; Cordain *et al.*, 2005; Lim *et al.*, 2005). Polysaccharide gums, such as gum arabic, guar, and locust bean gum, are commercial products used in foods and other materials (BeMiller, 1973; Phillips *et al.*, 1986; Trudso, 1988). They have weak gel properties, thus can be used as sol stabilizers and viscosity enhancers (Cui and Mazza, 1996; Chen *et al.*, 2006). Fedeniuk and

Biliaderis (1994) reported functional similarities (water binding abilities and rheological properties) between flaxseed mucilage and gum arabic in emulsions suggesting the possible substitution of gum arabic with mucilage. Flaxseed mucilage may serve as an emulsifying agent for chocolate milk (Manson and Hall, 1948). Flaxseed polysaccharides have been shown to function as a mucoadherent in the gastrointestinal tract as well as a drug-releasing, and a cryoprotective agent (O'Mullane and Hayter, 1993). Flaxseed mucilage has also been observed to possess a lubricating effect in the mouth and pharynx, making the polysaccharides a possible saliva substitute against xerostomia (Attstrom *et al.*, 1992).

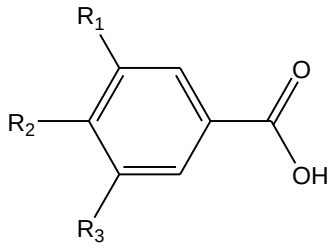
2.5.4 Minor components

2.5.4.1 Vitamins and minerals

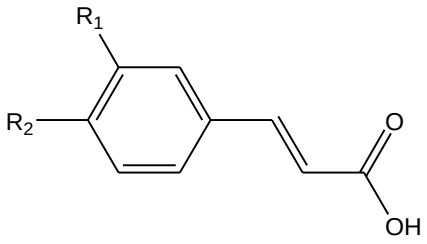
Flaxseed contains minor amounts of vitamins and minerals. Oomah *et al.* (1997) reported an average of 569 mg total tocopherols/ vitamin E, 0.5 mg vitamin C, and 5.3 mg total vitamins B per 100 g of whole flaxseed. Tocopherols are best known for its antioxidant activity (Anttolainen *et al.*, 1995). In addition, tocopherols also help lower blood pressure by promoting sodium excretion in the urine and lower the risk of Alzheimer's disease, heart disease, and some types of cancer (Morris *et al.*, 2005; Sen *et al.*, 2006). The major minerals in flaxseed are phosphorus, potassium, magnesium and calcium. Cadmium (Cd), a toxic element ubiquitous in soil, is also present in flaxseed (Holmgren *et al.*, 1993). The concentration of Cd in North American flax was reported to be higher than 0.3 mg/kg (Marquard *et al.*, 1990). As a comparison, the mean daily intake of Cd in Canadian diet was approximately 0.014 mg (Dabeka *et al.*, 1987). Excessive cadmium intakes (0.4 mg/week) subsequently led to cadmium accumulation in the kidney cortex and impaired kidney function (Nogawa *et al.*, 1987). Variations in the vitamin and mineral content in flaxseed has been linked to both plant genetics and environmental conditions. Tocopherol content reported in flaxseed is a function of accession, seed maturity, extraction protocol, growing region and seasonal conditions (Marquard *et al.*, 1977; Oomah *et al.*, 1997). Similarly, accumulation of Cd in flaxseed was reported to be specific to environment and genotype (Becher *et al.*, 1997; Li *et al.*, 1997; Grant *et al.*, 2000).

2.5.4.2 Polyphenols

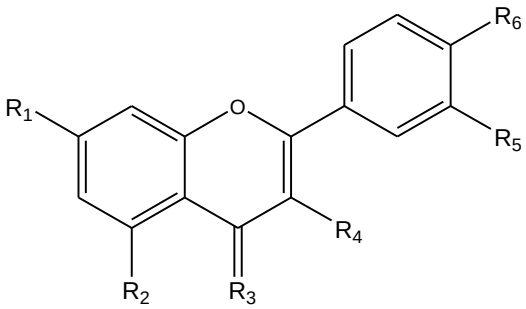
Polyphenols are defined as molecules possessing several hydroxyl groups on aromatic carbons (Manach *et al.*, 2004). Polyphenols act as a defense against pathogens and ultraviolet radiation, as well as, contribute colour to the plant as a means of attracting bees and other insects for pollination (Naczk and Shahidi, 2006). These plant secondary metabolites are classified based on the number of aromatic rings and their covalent bonds with other plant compounds. Three main classes of polyphenols in flaxseed are phenolic acids, flavonoids, and lignans (occurring mainly as secoisolariciresinol diglucoside or SDG) (Figure 2.4). Phenolic acids identified are ferulic acid, chlorogenic acid, and gallic acid, were reported at a concentration of 10.9, 7.5, and 2.8 mg/g, respectively, as well as traces of 4-hydroxybenzoic acid (Mazza, 2008). Flavones C- and O-glycosides are the major flavonoids found in flaxseed. Flaxseed is deemed as the richest source of SDG (6.1 to 13.3 and 11.7 to 24.1 mg/g in whole flaxseed and defatted flour, respectively) as it contains 75-800 times more than any other plant source (Mazur *et al*., 1996; Westcott and Muir, 1996). The concentrations of polyphenols in plants are greatly affected by genetics, environmental conditions (precipitation, sun exposure, and soil), processing conditions (industrial and domestic) and storage conditions (Manach *et al.*, 2004). The relative polyphenol composition is determined by maturity at harvest (Macheix *et al.*, 1990).



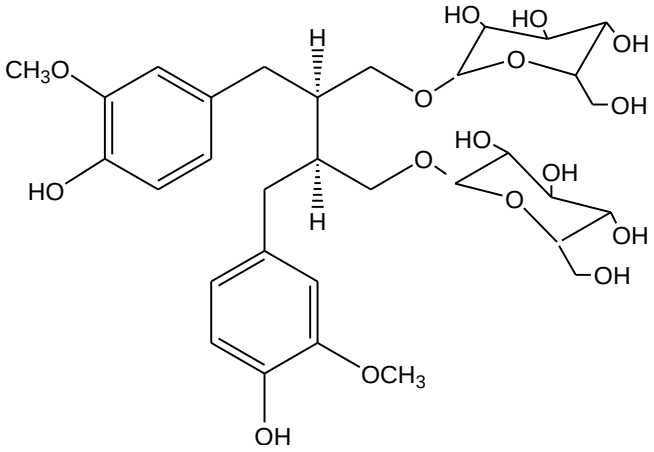
Hydroxybenzoic acid
$R_1 = R_2 = OH$, $R_3 = H$: Protocatechuic acid
$R_1 = R_2 = R_3 = OH$: Gallic acid



Hydroxycinnamic acid
$R_1 = OH$ Coumaric acid
$R_1 = R_2 = OH$: Caffeic acid
$R_1 = OCH_3$, $R_2 = OH$: Ferulic acid



Flavonoid
$R_1 = R_2 = R_4 = R_5 = R_6 = OH$, $R_3 = O$: Quercetin



Secoisolariciresinol diglucoside

Figure 2.4 Basic structures of phenolic compound present in flaxseed (Adapted from Pietta, 2000, Manach *et al*., 2004)

2.5.4.3 Plant antinutrients

Flaxseed contains antinutrients incuding cyanogenic glycosides, linatine, and phytic acid. Cyanogenic glycosides are glycosides of cyanohydrins, mostly in the form of β-linked D-glucosides (Vetter, 2000). Upon enzymatic hydrolysis of the glucosidic bond these compounds release unstable cyanohydrins that decompose in neutral or basic conditions to aliphatic ketones and hydrogen cyanide. These compounds exist in all flax tissues (Westcott and Muir, 2003). Linustatin and neolinustatin are the main cyanogenic glycosides of mature flaxseed (Westcott and Muir, 2003). The monoglucosides, linamarin and lotaustralin are found in leaves, flowers, developing embryos, and germinating seed. Stems and roots contain relatively low levels of cyanogenic glycosides (Niedzwiedz Siegien, 1998). Concentrations of cyanogenic glycosides in *Linum* spp. are mostly dependent on genotype, although variation dependent on maturity, development, and environment is also observed (Oomah *et al.*, 1992). The concentration of cyanogenic glycosides in young green flaxseeds can reach up to 5% of seed dry weight, whereas in mature seed, the amount was only around 0.1% (Butler and Conn, 1964). Linatine, an antivitamin B_6 compound was first reported by Kratzer *et al.* (1954), who observed vitamin B_6 deficiency syndromes (anemia, poor growth, loss of appetite, and nervous disorder with convulsions) in chicks fed a diet containing linseed meal. It occurs at about 10 mg/100 g in flaxseed meal (Mazza, 2008), with the greatest accumulation in the flaxseed cotyledon (Klosterman, 1967). Nugent (1974) however, observed that linatine occur in all parts of immature flax plants. Phytic acid is ubiquitous in foods derived from plant seeds (Daun *et al.*, 2003). Phytic acid makes up 2.3-3.3% of flaxseed dry seed weight, an amount comparable to that found in soybeans and peanuts (Reddy *et al.*, 1982). It is the primary form of phosphorus storage in seeds, representing about 60-90% of total seed phosphorus (Daun *et al.*, 2003). Phytic acid is an important flaxseed antinutrient, as it is able to bind divalent cations and hence, reduce mineral absorption, as well as, decrease protein and starch digestibility. The concentration of phytic acid in oilseeds is largely unaffected by accession and environment factors (Bhatty and Cherdkiatgumchai, 1990; Oomah *et al.*, 1996).

2.5.4.4 Sterols and pigments

Phytosterols are 28 to 29 carbon atom steroid alcohols that occur naturally in plants (Dutta *et al.*, 1996). They are involved in membrane permeability and fluidity, and therefore, are essential substances (Piironen *et al.*, 2000). β-Sitosterol and campesterol are the major sterols in flaxseed. It is considered common to find high level of β-sitosterol and low level of stigmasterol in seeds and meristematic tissues (Grunwald., 1975; 1980). Longer storage periods or germination, however, causes an increase level of stigmasterol level in the differentiated tissue of seedlings (Geuns, 1973; Stallaert and Geuns, 1994).

Carotenoids are fat-soluble polyisoprenoids (Zaripheh and Erdman, 2002). Carotenoids are synthesized as plant secondary metabolites and mainly localized in plastids (Faulks and Southon, 2005). Conjugated double bonds in carotenoids absorb visible light which contributes the distinctive colour of these plant pigments (Cunningham and Gantt, 1998). The major carotenoid pigment in flaxseed is lutein (Daun *et al.*, 2003), the only carotenoid present in the lens and retina of the eye, and chiefly responsible in photoprotection against retinal damage (Bone *et al.*, 1997; Mares-Perlman, 1999; Wooten *et al.*, 1999). Environmental factors, such as photoperiod, light intensity, and air temperature in addition to plant genetic, physiological, and biochemical attributes have been reported to be largely responsible for carotenoid accumulation in plants (Kopsell and Kopsell, 2008).

2.5.4.5 Cyclolinopeptides

By 2006, there have been 455 cyclic peptides (cyclopeptides) identified from 26 families, 65 genera, and 120 species of plants, particularly in Caryophyllaceae, Violaceae, and Rhamnaceae (Tan and Zhou, 2006). Cyclopeptides are composed of 2 to 37 amino acids, including proteinogenic or non-proteinogenic amino acids. Most of the cyclic peptides observed to date are bioactive, including acting as immunostimulants, cytotoxic agents, sedatives, antifungals, antibacterials, antimycobacterials, antimitotics, and antiplasmodial agents (Tan and Zhou, 2006). Potential application of cyclopeptides as cryptands was reported by Ghadiri *et al*. (1993). Cyclopeptides with even number of alternating L and D amino acids are capable to self-assemble and form hollow tubular core structures through intermolecular hydrogen bonding. The resultant nanotubes, together with C=O and N-H functional groups in turn provide a binding cavity suitable for binding other molecules. Cyptands have found applications in a wide range of

products. For example, cyclodextrins, a group of cyclic oligosaccharides are used for flavour delivery and protection of volatile compounds (Hedges, 1998), as well as, for removing undesirable compounds from foods (Szejtli, 1998). In addition to improve solubility and availability of guest molecules (Rajewski and Stella, 1996), cyclodextrins are also used in the pharmaceutical industry to mask medicine flavours (Frömming and Szejtli, 1994). In cosmetics and perfumes, cyclodextrins preserve odor by controlling the release of aromatic oils (Prasad *et al*, 1999), whereas, in environmental science, cyclodextrins can be used as additives in environmental bioassays that enhance bioavailability and solubility of contaminants during the process of soil remediation (Hajdu *et al*, 2011).

Based on their skeletons and distributions in plants, cyclic peptides from *L. usitatissimum* (Linaceae), called cyclolinopeptides (CLs) are categorized as homomonocyclopeptides (type VI, Caryophyllaceae-type) (Tan and Zhou, 2006). Cyclopeptides classified in this category are those that exist as a single ring containing peptides bonds of 2 or between 5 to 12 protein or non-protein α–amino acids (Tan and Zhou, 2006). In total, 168 caryophyllaceae-type cyclopeptides have been discovered in ten families, including Annonaceae, Caryophyllaceae, Rutaceae, Verbenaceae, Euphorbiaceae, Labitacae, Araliaceae, Phytolaccaceae, Schizandraceae, and Linaceae. There is currently no information about the biosynthesis of CLs within the Linaceae family. However, studies involving Caryophyllaceae family (*Pseudostellaria heterophylla* (Jia *et al*., 2006) and *Saponaria vaccaria* (Condie *et al*., 2011) indicated the evidence of ribososome-dependent biosysnthesis of Caryophyllaceae-type cyclopeptides.

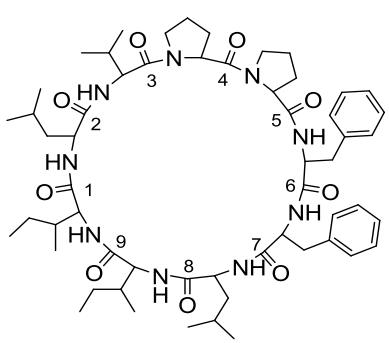
The discovery of CLs was described by Kaufmann and Tobschirbel (1959) who isolated CLA (**1**, Table 2.2) from precipitated "slime" obtained as a byproduct in the processing of crude flaxseed oil. Since then, other CLs have been discovered from flaxseed and root extracts (Weygand, 1986; Morita *et al.,* 1997; Morita *et al.,* 1999; Matsumoto *et al.,* 2001). CLs are hydrophobic in nature and composed of eight or nine amino acid residues with molecular weights of approximately 1kDa (Morita and Takeya, 2010). The primary structures of fully elucidated CLs are summarized in Figure 2.5. The new naming designations of CLs are presented together with the literature nomenclature. In contrast with the older system which names CLs alphabetically based on the order of discovery, the newer system names peptides based on the location and oxidation state of methionine (Met) in the respective parent peptide. The National

Center for Biotechnology Information (NCBI) gene sequence accession #AFSQ01016651.1 contains the embedded sequences of **1** (ILVPPFFLI), **2** (MLIPPFFVI), and **8** (MLVFPLFVI) (Figure 2.6), whereas NCBI gene AFSQ01025165.1 contains the embedded sequences of **5** (MLLPFFWI), **11** (MLMPFFWV) and **14** (MLMPFFWI) (Figure 2.7) (Sayers *et al*., 2009). Picur *et al*. (1998) isolated CLX [*cyclo*-(Pro-Pro-Phe-Phe-Leu-Leu-X)] from milled cake of *L. album*, where X is a non-protein amino acid, *N*-methyl-4-aminoproline.

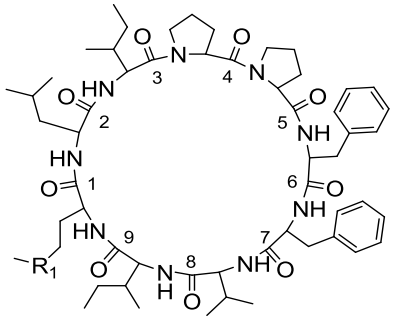
The location of CLs deposition in flaxseed was investigated by Gui *et al.* (2012a). Levels of CLs in three flaxseed portions (mucilage, seed coat, and cotyledon) were compared with those in whole seeds. CLs **1-3, 6, 9, 12, 15** were observed in seed coat and cotyledon in total concentration of 123.1 ± 4.8 and 291.9 ± 8.2 µg/g, respectively (Gui *et al.,* 2012a). No peptide was detected in the water-soluble mucilage. Specifically, CLs are stored in oleosomes or oil bodies (Gui *et al.,* 2012b), the main organelle for oil storage in plant seed (Huang, 1996). Oleosomes are comprised of approximately 97.7% triacylglycerides, 1.3% protein, 0.9% phospholipids, and 0.1% fatty acids (Tzen *et al*., 1997), with diameter ranges from 0.5 to 2.0 µm (Gui *et al.,* 2012b).

Table 2.2 Cyclolinopeptides from *Linum usitatissimum* L.

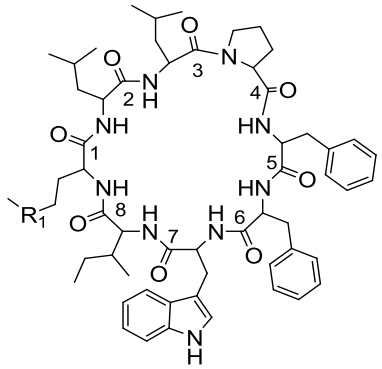
Old name	New name	Amino acid sequence	Chemical Formula	Molecular weight	Number
CLA	CLA	*cyclo*-(Ile-Leu-Val-Pro-Pro-Phe-Phe-Leu-Ile)	$C_{57}H_{85}N_9O_9$	1040.34	**1**
CLB	1-Met-CLB	*cyclo*-(Met-Leu-Ile-Pro-Pro-Phe-Phe-Val-Ile)	$C_{56}H_{83}N_9O_9S$	1058.38	**2**
CLC	1-Mso-CLB	*cyclo*-(Mso-Leu-Ile-Pro-Pro-Phe-Phe-Val-Ile)	$C_{56}H_{83}N_9O_{10}S$	1074.38	**3**
CLK	1-Msn-CLB	*cyclo*-(Msn-Leu-Ile-Pro-Pro-Phe-Phe-Val-Ile)	$C_{56}H_{83}N_9O_{11}S$	1090.38	**4**
CLD’	1-Met -CLD	*cyclo*-(Met-Leu-Leu-Pro-Phe-Phe-Trp-Ile)	$C_{57}H_{77}N_9O_8S$	1048.34	**5**
CLD	1-Mso-CLD	*cyclo*-(Mso-Leu-Leu-Pro-Phe-Phe-Trp-Ile)	$C_{57}H_{77}N_9O_9S$	1064.34	**6**
CLD’ $_{Msn}$	1-Msn-CLD	*cyclo*-(Msn-Leu-Leu-Pro-Phe-Phe-Trp-Ile)	$C_{57}H_{77}N_9O_{10}S$	1080.34	**7**
CLE’	1-Met-CLE	*cyclo*-(Met-Leu-Val-Phe-Pro-Leu-Phe-Ile)	$C_{51}H_{77}N_8O_8S$	961.26	**8**
CLE	1-Mso-CLE	*cyclo*-(Mso-Leu-Val-Phe-Pro-Leu-Phe-Ile)	$C_{51}H_{77}N_8O_9S$	977.26	**9**
CLJ	1-Msn-CLE	*cyclo*-(Msn-Leu-Val-Phe-Pro-Leu-Phe-Ile)	$C_{51}H_{77}N_8O_{10}S$	993.26	**10**
CLF’	1-Met, 3-Met-CLF	*cyclo*-(Met-Leu-Mso-Pro-Phe-Phe-Trp-Val)	$C_{55}H_{73}N_9O_8S_2$	1051.50	**11**
CLF	1-Mso, 3-Mso-CLF	*cyclo*-(Mso-Leu-Mso-Pro-Phe-Phe-Trp-Val)	$C_{55}H_{73}N_9O_{10}S_2$	1084.35	**12**
CLI $_{isomers}$	1-Mso, 3-Met-CLF	*cyclo*-(Met-Leu-Mso-Pro-Phe-Phe-Trp-Val)	$C_{55}H_{73}N_9O_9S_2$	1068.35	**13**
CLG’	1-Met, 3-Met-CLG	*cyclo*-(Met-Leu-Met-Pro-Phe-Phe-Trp-Ile)	$C_{56}H_{75}N_9O_{10}S_2$	1066.38	**14**
CLG	1-Mso, 3-Mso-CLG	*cyclo*-(Mso-Leu-Mso-Pro-Phe-Phe-Trp-Ile)	$C_{56}H_{75}N_9O_{10}S_2$	1098.38	**15**
CLH $_{isomers}$	1-Mso, 3-Met-CLG	*cyclo*-(Mso-Leu-Met-Pro-Phe-Phe-Trp-Ile)	$C_{56}H_{75}N_9O_9S_2$	1082.38	**16**



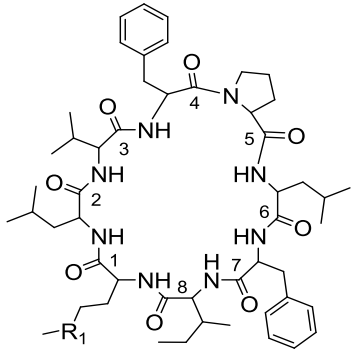
CLA (1)



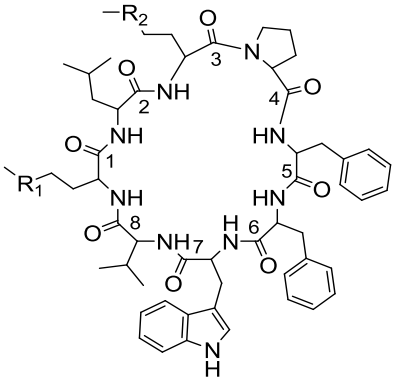
1-Met CLB (2), R1 = S
1-Mso CLB (3), R1 = SO
1-Msn CLB (4), R1 = SO_2



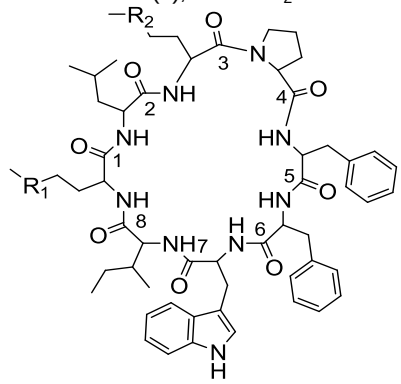
1-Met CLD (5), R1 = S
1-Mso CLD (6), R1 = SO
1-Msn CLD (7), R1 = SO_2



1-Met CLE (8), R1 = S
1-Mso CLE (9), R1 = SO
1-Msn CLE (10), R1 = SO_2



1-Met,3-Met CLF (11), R1 = R2 = S
1-Mso,3-Mso CLF (12), R1 = R2 = SO_2
1-Met,3-Mso CLF (13), R1 = S, R2 = SO
1-Mso,3-Met CLF (13), R1 = SO, R2 = S



1-Met,3-Met CLG (14), R1 = R2 = S
1-Mso,3-Mso CLG (15), R1 = R2 = SO_2
1-Mso,3-Met CLG (16), R1 = SO, R2 = S
1-Met,3-Mso CLG (16), R1 = S, R2 = SO

Figure 2.5 Cyclolinopeptides from *Linum usitatissimum* L.

Coding sequence of #AFSQ01016651.1

ATGGCTGTTG TGTCCTCTCT GGCTCTGACC ACTAGCCTAG TTGCTACCGC CGCCGGCCGT
AATAATAATG CCTTCCCACC ATCCTCCTCC AGGAACAACA AGGCACCAGC AGACCTTTTC
ATTACTCCCA AGACAACAAC AACAGTGAAA GCAGCAGCTG TCTCATGCAA ACGTCCCTA
CCGAAAGGAG CAGTTGCTGC TGCTACTAGT ACCTTGTCTC CTATTTCTGG AAAGGATGGC
GGCCTCCGCA ACCAGGAGGA GAGCGATGGT ATGTTGGTAT TCCCCTTATT TATATTCGGC
AAGGAAGGTA GTCAGGACAA GTATAATGGA GCAGCTGCCC TCCGCGACCA GGAGGAGAGC
GATGGTATGT TGATCCCCCC CTTCTTTGTC ATATTCGCA AGGAAGGTTG TCAGGATATC
GGCCACAAGT ATAATAATGC CGCAGCAGCT GGCGCCCTCC GCGACCAGGA GGAGAGCGAT
GGTATAGTGG TCCCCCCCTT CTTTCTCATA TTCGGCAAGG AAGGTAGTCA GGACAAGTAT
AATGCAGCAG CAGCTGGCGG CCTCCGCGGC AAGGAGCAGC AGGGTGACAA GATGGCGGCT
GGAGCTGAGA ATTAG

Translation of sequence #AFSQ01016651.1

MAVVSSLA LTTSLVAT AAGRNNNA FPPSSSRN NKAPADLF ITPKTTTT VKAAAVSC KRPYPKGA
VAAATSTL SPISGKDG GLRNQEES DG**MLVFPL** **FI**FGKEGS QDKYNGAA ALRDQEES DG**MLIPPF**
FVIFGKEG CQDIGHKY NNAAAAGA LRDQEESD G**ILVPPFF** **LI**FGKEGS QDKYNAAA AGGLRGKE
QQGDKMAA GAEN_

Figure 2.6 Gene sequence and amino acid translation of #AFSQ01016651.1 encoding CLA (**1**, ILVPPFFLI), 1-Met-CLB (**2**, MLIPPFFVI), and 1-Met-CLE (**8**, MLVFPLFI)

Coding sequence of AFSQ01025165.1

ATGGCTCTTG TGTCCTCTCT GGCTCTGACC ACTAGCCTAG TTGCTACCGC CGCCGGCCGT
AATAATAATG CCTTCCCACC ATCCTCCTCC AGGAACAACA AGGCACCAGC AGACCTTTTC
ATTACTCCCA AGACAACAAC AACAGTGAAA GCAGCAGCTC TCTCATGCAA ACGTCCCTAC
CCGAAAGGAG CAGTTGCTGC TGCTACTAGT ACCTTGTCTC CTATTTTCTGG AAAGGATGGC
GGCCTCCGCA ACCAGGAGGA GAGCGATGGT ATGTTGGTCT TCCCCTTATT TATATTCGGC
AAGGAAGGTA GTCAGGACAA GTATAATGGA GCAGCTGCCC TCCGCGACCA GGAGGAGAGC
GATGGTATGT TGATCCCCCC CTTCTTTGTC ATATTCGGCA AGGAAGGTTG TCAGGATATC
GGCCACAAGT ATAATAATGC CGAAGCAGCT GGCGCCCTCC GCAACCAGGA GGAGAGCGAT
GGTATACTGG TCCCCCCCTT CTTTCTCATA TTCGGCAAGG AAGGTAGTCA GGACAAGTAT
AATGCAGCAG CAGCTGGCGG CCTCCGCGGC AAGGAGCAGC AGGGTGACAA GATGGCGGCT
GGAGCTGAGA ATTAG

Translation of sequence AFSQ01025165.1

MAAASSLA LATASLVA TGAGGRNN AFLPSKNK TPNLFLNP NKTTSSTV KAVVSSSS CKRPYPKG
DASLFLGI DDVFGKDA VAGHDNDQ DAASGQEM AADD**MLMP** **FFWI**FGKE GQQQEAEE SSDD**MLMP**
FFWIFGKE GQQQEAES SDD**MLLPF** **FWI**FGKEG QQQEAESS DD**MLMPFF** **WI**FGKQQQ QQGESSDD
MLMPFFWV FGKQGDNN KGDAVEAI LKN_

Figure 2.7 Gene sequence and amino acid translation of AFSQ01025165.1 encoding 1-Met-CLD (**5**, MLLPFFWI), 1-Met,3-Met-CLF (**11**, MLMPFFWV) and 1-Met,3-Met-CLG (**14**, MLMPFFWI)

2.5.4.5.1 Biological activities of cyclolinopeptides

CLs are biologically active. Kessler *et al.* (1986) described the cytoprotective effect of **1** on hepatocyte which was revealed by the inhibition of cholate. The tripeptide sequence -Phe-Phe-Pro was proposed to impart the observed cytoprotective ability of **1** by suppressing the hepatocyte transport system. Strong hydrophobic nature of **1** is linked to its antimalarial activity against human parasite *Plasmodium falciparum* in *in vitro* tests (Bell *et al*., 2000). CLs are also reported to exhibit immunomodulatory activities (Morita *et al.*, 1999; Morita and Takeya, 2010). Wieczorek, *et al.* (1991) compared the immunosuppressive activity of **1** and cyclosporine A (CsA). Tests involving *in vitro* and *in vivo* determination of primary and secondary humoral response using the plaque-forming cell test, the delayed-type hypersensity test, skin-allograft rejection, and the graft-vs. –host reaction showed that CLA produced similar biological responses to CsA. Compound **1** was also found to temper haemolytic anemia in New Zealand Black mice and post adjuvant polyarthritis in rats, as well as influence human lymphocyte proliferation *in vitro*. Morita *et al.* (1997) reported a comparable inhibition activity of concanavalin-A induced proliferation of human peripheral blood lymphocyte by **2** to that of CsA (Wieczorek, *et al.*, 1991). Additionally, CLs **2** and **9** have also been shown to possess moderate inhibitory effect on concanavalin-A induced mouse lymphocyte proliferation (Morita *et al.*, 1997). The immunosuppressive ability of CLs is proposed to be related to the -Pro-Xxx-Phe- sequence, where Xxx is a hydrophobic, aromatic, or aliphatic amino acid (Picur *et al*., 2006). Various studies comparing immunosuppressive activity of **1** against its chemical analogs have proved high efficacy of **1** (Siemion *et al*., 1999; Benedetti *et al*., 1999; Picur *et al*., 2006).

Potential of CLs as future immunosuppresive drugs was evaluated by administration of relatively large doses of **1** followed by determining the immune response and toxicity to rats and mice (Wieczorek, *et al.*, 1991). Intraperitoneal administration of CLA at the doses of 3 g/kg to rats and 4 g/kg **1** (in olive oil, 2% gelatin solution, and intralipid) to mice, as well as 230 mg/kg intravenous injection to mice was deemed to be nontoxic. Additionally, rats treated with **1** through oral administration with 15, 45, and 135 mg/kg for 13 weeks survived the experiment without harm. On the other hand, CLs potential as nanodelivery device for biomedical application was investigated by observing biomolecular interactions of **1** with human serum albumin (HSA) (Rempel *et al*., 2010). HSA, the most abundant protein in blood plasma occurs at concentration of 40 $mg \cdot mL^{-1}$ (Rempel *et al*., 2010). As a transport protein, HSA regulates

intercellular fluxes by interacting with organic and inorganic compounds (Ascenzi *et al*., 2006). Binding of **1** to HSA demonstrated CLs fate in physiological system following consumption of flax products. Interaction between **1** and HSA was described as endothermic. Binding affinity appeared to correlate positively with temperature (Rempel *et al*., 2010). This entropy-driven reaction suggested disassociation of the solvation complex, consisting of dimethyl sulfoxide (DMSO), **1**, and H_2O and hydrophobic binding of **1** to HSA. Interaction of **1** to HSA is important for potential of CLs as drug ingredient or as nanodelivery device used in nutraceutical and biomedical applications (Rempel *et al*., 2010).

2.5.4.5.2 Oxidation of cyclolinopeptides

In contrast to the stability of flax oil in the intact seed, other flaxseed products, including oil, cake and meal (Wanasundara and Shahidi, 1998; Wiesenborn *et al.*, 2005) are subject to rapid oxidation. The flavour of fresh, unrefined flaxseed oil is usually described as a mild, nutty and pleasant flavour (Wiesenborn *et al.*, 2005). Other contributors to the flavour chemistry of flax oil are seed variety, quality, handling, processing, and storage, as well as, phytochemical constituents of the flax oil (Brühl *et al.*, 2007; 2008). Brühl *et al.* (2007) reported the oxygenation of Met-containing peptides in flaxseed oil stored over 150 days. Several CLs, including **2**, **13**, and **16** were absent after long-term storage. Oxidation of Met during long-term storage of flax oil is linked to the production of a bitter off-flavour (Brühl *et al.*, 2007). Bitterness is affected by the structure, polarity, and hydrophobicity of peptides (Kim *et al.*, 2008). Ney (1971) pointed the high probability of peptides with M. W. less than 6 kDa and hydophobicity values greater than 1400 cal in contributing bitter flavour. As well, the study reported that presence of certain amino acids such as Leu, Pro, Phe, Tyr, Ile or Trp might also impart bitterness in peptides. A study by Brühl *et al.* (2007) identified **9** as the major principle of the bitterness in aging flax oil. Exposure to oxygen and heat was believed to be the major contributing causes of Met oxidation and increased level of the oxidized bitter peptide, **9**. Oxidization of Met to Mso and Msn can also be achieved through the action of biological and chemical agents (Cuq *et al.*, 1973; Shechter, 1986). Vogt (1995) reported that Met conversion to Mso was induced by the superoxide anions during oxidative metabolism in biological systems. Similarly, Shechter (1986) found that Met was effectively oxidized to Mso by hydrogen peroxide in an acid environment. In a further study

conducted by Brühl *et al.* (2008) the accumulation of **9** was observed in 21 flax accessions grown in Suhr (AG, Switzerland). Concentrations ranging from 0 to 53 mg/kg of seed were reported. A parallel experiment comparing HPLC analysis and sensory evaluation a positive correlation was determined between the detected level of **9** in crude pressed-oil and the degree of oil bitterness (Brühl *et al.*, 2008). However, the report also indicated a possibility of other substances in flax contributing to the overall the bitter flavour.

2.5.4.5.3 Isolation of cyclolinopeptides

Various protocols for isolation of CLs from flax products have been reported. The first CL discovered was isolated from the precipitation of flax oil after extraction and settling (Kaufmann and Tobschirbel, 1959). Other means to isolate CLs are through extraction of defatted flaxseed, roots, and meal with hot methanol (MeOH). The extract was subjected to polystyrene column eluting with increasing concentration of MeOH in water (H_2O) and the bound peptides were removed with MeOH (100%, v/v). The resulting extract was loaded on silica gel column and washed with decreasing concentration of chloroform (CH_3Cl) in MeOH. The peptide-containing fraction 10-15% CH_3Cl (v/v) in H_2O, subsequently subjected reversed phase HPLC yielded 0.007% **1**, 0.0002% **2**, 0.0037% **3**, 0.0015% **6**, 0.0058% **9**, 0.0008% **12**, 0.0024% **15**, 0.0002% **16**, and 0.00007% **13** (Morita *et al.*, 1997a;b; 1999; Matsumoto *et al.*, 2001a;b; 2002). Solvent extraction of flaxseed with acetone, followed with hydrolysis using 10% sodium hydroxide (Stefanowicz, 2001) has also yielded CLs though no further separation on individual CLs was reported using this method. Later, Reaney *et al*. (2009) proposed a commercial extraction and concentration of CLs using either solid-liquid or liquid-liquid extraction of freshly pressed flax oil. The proposed method enabled higher peptides recovery ratio when compared to other published protocols. For example, protocol by Reaney *et al*. (2009) recovered 146 mg of crude CLs from 100 g of flax oil, from which was purified 16.7 mg of **9**. A yield almost 1.5-fold greater than reported by Brühl *et al.* (55 mg of crude peptides from 100 g of flax oil, from which 11 mg of **9** was recovered) (Brühl *et al.*, 2007). On the other hand, production of CLs can be achieved through chemical synthesis (Wiezorek *et al*., 1991). The synthetic method however, is costly and producing a low yield of peptide.

CHAPTER 3
RAPID REVERSED PHASE LIQUID CHROMATOGRAPHY SEPARATION OF CYCLOLINOPEPTIDES WITH MONOLITHIC AND MICROPARTICULATE COLUMNS

3.1 Abstract

Three monolithic C_{18}-bonded silica gel columns i.e. Chromolith® SpeedROD (CSR), Chromolith® Performance (CP), and Chromolith® High Resolution (CHR), MerckKGaA Darmstadt, Germany and two particle-based columns i.e. ZORBAX Eclipse XDB-C_{18} (ZEX), Agilent and POROS R1/20 (POR), Applied Biosystems were compared for their performance in separating a mixture of flaxseed cyclolinopeptides (CLs). Gradient mobile phases of acetonitrile and water were optimized for each column. The performance of CHR column in profiling CL standards, measured as the resolution of individual CL, selectivity, and peak asymmetry exceeded the performance of traditional particle-packed columns and the other monolithic columns. The profiling of CLs in aqueous methanolic flaxseed extract was optimized for high-throughput analysis. A total analysis time of 1.5 min at a flow rate of 3.0 $mL \cdot min^{-1}$ was achieved on a CSR column. Injection of over 2,000 methanol extracts of flaxseed on a CSR column had no impact on backpressure or resolution of a standard CL mixture.

3.2 Introduction

Flax (*Linum usitatissimum* L.) seeds contain natural hydrophobic cyclic peptides (cyclolinopeptides/CLs), comprising eight or nine amino acid residues with molecular weights of approximately 1kDa (Morita and Takeya, 2010). Similar with flax oil which is readily oxidized due to its high content of polyunsaturated fatty acids, methionine- containing CLs such as **2** and **8** are known to oxidize as flaxseed oil ages (Brühl *et al.*, 2007). Methionine (Met) can be oxidized

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to methionine sulfoxide (Mso) and further to methionine sulfone (Msn) *via* chemical and/or biological means (Cuq *et al*., 1973; Shecter, 1986; Vogt, 1995). Exposure to oxygen and heat are considered to be the major contributing causes of methionine oxidation. This makes isolation and purification of methionine-containing peptides vital because the reversible methionine oxidation and reduction is a well-established molecular mechanism for cellular regulation (Hoshi and Heinemann, 2001).

Separation and identification of CLs from flaxseed oil has been achieved through conventional reversed phase-HPLC using particle packed columns [250 mm × 4 mm, 5 μm, LiChrospher 100 RP-18 column (MerckKGaA) and ZEX (Agilent)] that employ lengthy methods which are not suitable or convenient for high-throughput analysis (Brühl *et al.*, 2007; Reaney *et al*., 2009). Shorter elution times are possible by reducing column length and/or increasing the flow rate (Spoof and Meriluoto, 2002). Shorter columns however have lower resolution while higher flow rate increases backpressure and potentially lowers column resolution. Use of a shorter column with small particle size (<3 μM) operating at high backpressure is one approach that may yield rapid separation (Yang *et al*., 2006).

Attempts to improve chromatographic stationary phases led to the introduction of monolithic macroporous columns in the early 1990s to allow for high-speed separation of analytes without compromising column efficiency (Sveč and Fréchet, 1992). These chromatographic polymer-based media possess different structures from conventional silica packed columns. Monolithic columns contain solid porous rod comprising interconnected skeletons and flow paths that make up the pores (Josic *et al*., 2001). They are characterized by mesopores and macropores that offer significantly greater porosity, greater permeability and higher surface area compared to conventional columns packed with spherical beads (Spoof and Meriluoto, 2002). The small skeleton and large through-pores of monolithic columns results in improved flow-independent mass transfer properties and separation efficiency when compared to particle-based columns. Hence, monolithic columns possess low hydraulic resistance (Plieva *et al*., 2009) and can withstand high flow rates, a condition usually associated with high back pressure problems. Monolithic columns are composed of synthetic organic material (acrylate, polystyrene, acrylamide), inorganic material (silica), or natural polymers (cellulose) (Spoof and Meriluoto, 2002; Josic *et al*., 2001) that can be cast in the forms of disks, rods, or tubes. Monolithic columns are commercially available through a number of manufacturers (Plieva *et al*.,

2009) and are used for fast separation of biological macromolecules, smaller biomolecules, preparative isolation, and separation of diastereomers (Spoof and Meriluoto, 2002). Perfusion columns that contain a special matrix geometry that has very large (400-800 nm) pores connected to a network of smaller (30-100 nm) pores (Synder *et al*., 2010) have been developed to meet the need for rapid separations. A combination of diffusion and flow of the mobile phase through the pores carries solutes into and out of the perfusion matrix. This unique flow pattern is advantageous in aiding the stationary-phase mass transfer and reducing band broadening especially for large molecules such as proteins even at higher flow rates.

The primary objective of this work was to develop an HPLC method for high-throughput screening of CLs in flaxseed varieties. To achieve this, the resolution, selectivity, capacity factor and symmetry of five commercial chromatographic columns were evaluated. A mixture of four CL standards (**2-4** and 1-Abu-CLB) was used for all chromatographic investigations. Column longevity of CSR was determined while screening flaxseed varieties to determine CL content.

3.3 Experimental

3.3.1 Reagents and samples

Water (H_2O) was purified to 18.2 MΩ cm on a Milli-Q Integral system (Millipore, Molsheim, France). Chemicals used in the conduct of this study are listed in Appendix A. A standard of **2** was obtained by solvent partitioning of flaxseed oil using 95% aq. ethanol (1:1, v/v). The organic layer was concentrated and was subjected to flash column chromatography (FCC) on silica gel 60 (40-63 µm particle size, EMD Chemicals). Sequential elution was performed using: (a) ethyl acetate (EtOAc, 25%, v/v) in *n*-hexane; (b) EtOAc (50%, v/v) in *n*-hexane; (c) EtOAc (75%, v/v) in *n*-hexane; (d) EtOAc (100%, v/v); (e) MeOH (5%, v/v) in dichloromethane (CH_2Cl_2); (f) MeOH (7.5%, v/v) in CH_2Cl_2; and (g) MeOH (10%, v/v) in CH_2Cl_2. A crude mixture comprising cyclolinopeptides **1** and **2** (fraction d) was extracted with diethyl ether, the combined extract was filtered, and was subsequently concentrated using a rotary evaporator. The resulting crude extract was dissolved in MeOH (1:8, w/v) and was filtered. Compound **2** was purified from the mixture on a HPLC system (BioCAD® Sprint) using a reversed phase preparative column (GL Sciences Inc. Inertsil Prep – ODS, 10 μm particle size silica gel, 250 mm × 30 mm I.D). The mobile phase consisted of H_2O–CH_3CN (45:55 for 9 min, to 10:90 in 30 min, and to 45:55 in 1 min). Standards of **4** and **5** were extracted from

cyclolinopeptide-laden silica gel (Reaney *et al.*, 2009). Firstly, the cyclolinopeptide-containing silica gel was extracted with *n*-hexanes (1:2, w/v) with periodic stirring (30 min) to remove residual oil. The resulting defatted silica was eluted with: (a) EtOAc (50%, v/v) in *n*-hexanes and (b) MeOH (10%, v/v) in CH_2Cl_2. Fraction b was concentrated and the resulting residue was taken in EtOAc (500 mL) and was washed with a saturated solution of sodium bicarbonate (500 mL) and brine. The organic phase was dried, filtered, and subjected to FCC on silica gel 60 eluting with: (a) EtOAc (80%, v/v) in *n*-hexane; (b) EtOAc (100%, v/v); (c) MeOH (2.5%, v/v) in CH_2Cl_2; (d) MeOH (5%, v/v) in CH_2Cl_2. Fraction d contained a mixture of **4** and **5,** and was subsequently purified using preparative HPLC as described above.

Compound 1-Abu-CLB was prepared by reductive elimination of sulfur from the methionyl residue using Raney Nickel (16 h at 240 °C) and was purified using an Agilent 1200 series HPLC system. The mobile phase consisted of H_2O–CH_3CN (39:61 for 3 min, to 10:90 in 0.5 min, to 39:61 in 0.5 min, and to 39:61 in 1 min) at a flow rate of 5.0 $mL \cdot min^{-1}$. A Chromolith® Semiprep (100 mm × 10 mm I.D, Merck KGaA Darmstadt) column was used in this purification. The standards were subjected to HPLC, High Resolution HPLC/ESI–MS and HPLC/ESI–MS/MS analyses (Appendix B). Flaxseed extract was obtained by 80% aq. MeOH (1:8, w/v) extraction of ground (degummed) flaxseed at 60 ºC for 2 h, into which an internal standard, 1-Abu-CLB was added. The extract was oxidized with hydrogen peroxide to convert all Met residues to similar chemical forms, that is, Mso. The reaction was quenched with sodium thiosulfate to prevent over oxidation of Met to Msn.

3.3.2 HPLC apparatus

All chromatographic separations were performed on Agilent 1200 series HPLC system (Agilent Technologies Canada, Mississauga, ON) equipped with a quaternary pump, autosampler, photodiode-array detector (wavelength range 190-300 nm), and a degasser. The column compartment temperature was 23 ºC, unless otherwise stated. Eluting peaks were detected at wavelengths of 214 nm with a 10 nm bandwidth against a reference signal at 300 nm with a 10 nm bandwidth using Chemstation LC 3D™ system software (Agilent Technologies Canada Inc., Mississauga, ON). Injection volume was maintained at 10 µL for all investigations. The mobile phase consisted of a gradient of water-acetonitrile as detailed in section 3. A flow rate of 2.0 $mL \cdot min^{-1}$ was used unless otherwise stated. The HPLC/ESI–MS and HPLC/ESI–MS/MS were

performed on an Agilent HPLC 1200 series directly connected to a Bruker microTOF-Q II Mass Spectrometer (Hybrid Quadrupole–TOF MS/MS, Bruker Daltonik GmbH, Bremen, Germany) with Apollo II ESI ion source. The MS-conditions were: Nebulizer gas (N_2) 58 psi, dry gas (N_2) 8 $L \cdot min^{-1}$, dry temperature 200 °C, HV capillary 4500 V, and collision energy 30 eV. The MS^n acquisition was obtained for the mass range 50 to 2000 Da. A Chromolith FastGradient RP-18e column (3 μm particle size silica, 50 mm × 2.0 mm I.D., Merck KGaA, Darmstadt, Germany) was used to separate compounds prior to MS analysis. The mobile phase consisted of a linear gradient of 0.1% formic acid in H_2O and 0.1% formic acid in CH_3CN (60:40 for 2 min, to 10:90 in 8 min, to 60:40 in 0.5 min, to equilibration for 5.5 min) at a flow rate of 0.40 $mL \cdot min^{-1}$.

3.3.3 Stationary phase

Three monolithic C_{18}-bonded silica rod columns (CSR, 50 mm × 4.6 mm I.D; CP, 100 mm × 4.6 mm I.D; CHR, 100 mm × 4.6 mm I.D) and a particle-packed column (POR, 100 mm × 4.6 mm I.D) were tested for their utility in separating a CL mixture. For reference purposes, a particle-packed column (ZEX, 150 mm × 4.6 mm I.D) was also employed. The main characteristics of the columns studied are provided in Table 3.1.

3.3.4 Sample preparation

Standards of **2–4** and 1-Abu-CLB were each weighed (MSA225S000DU, Sartorius, Germany) and dissolved in methanol (5.0 mL) in separate volumetric flasks to make stock solutions of 0.4 $mg \cdot mL^{-1}$. The flasks were subsequently stoppered and sealed with Parafilm™ (Pechiney Plastic Packaging, Chicago, IL) to limit solvent evaporation. A mixture of CL was prepared by combining 300 μL of each CL stock solution giving a total CL concentration of 0.4 $mg \cdot mL^{-1}$ with each CL being 0.1 $mg \cdot mL^{-1}$.

3.3.5 Calculations of chromatographic parameters

Chemstation LC 3D™ system software (Agilent Technologies Canada Inc., Mississauga, ON) was used to calculate peak parameters.

$$R = 1.18\,(t_R1 - t_R2)/\,(W_{1/2}\,1 + W_{1/2}\,2) \qquad (1)$$

$$k' = (t_R - t_0)/\,t_0 \qquad (2)$$

$$\alpha = (t_R2 - t_0)/\,(t_R1 - t_0) \qquad (3)$$

where t_R is the retention time, $W_{1/2}$ is peak width at half height, and t_0 is column void volume. Eqs. (1), (2), and (3) were used to calculate peak resolution, capacity factor, and selectivity, respectively.

Table 3.1. Characteristics of the chromatographic columns compared in this study

	Chromolith® SpeedROD	**Chromolith® Performance**	**Chromolith® High Resolution**	**ZORBAX Eclipse XDB-C_{18}™**	**POROS R1/20**
Macropore size (µm)	2.0	2.0	1.15	_	_
Mesopore size (nm)	13	13	15	8	50 – 1000
Particle size (µm)	_	_	_	5.0	20
Specific surface area (m^2/g)	300	300	250	180	
Material	Silica based monolithic skeleton	Silica based monolithic skeleton	Silica based monolithic skeleton	Silica particles	Poly (styrene-divinylbenzene) particles
Manufacturer	Merck KGaA	Merck KGaA	Merck KGaA	Agilent	Applied Biosystems

3.4 Results and discussion

3.4.1 Study of cyclolinopeptide standards mixture using monolithic and microparticulate columns

Compounds **2–4** and 1-Abu-CLB were chosen because they differ by only the oxidation state and/or presence of the Met residue. Moreover, 1-Met-CLB (**2**) and 1-Msn-CLB (**4**) crystallize readily and, based on NMR observation, only occur in a *cis-trans* prolyl configuration (Schatte *et al*., 2011; Jadhav *et al*., 2011). Chromatographic separation of the CL mixture was performed on a commercial particle-packed ZEX column using water and acetonitrile gradients as summarized in Table 3.2. Within the 30 min analysis, the peptides were effectively separated with **3**, **4**, 1-Abu-CLB, and **2** having retention times of 7.6, 10.4, 14.5, and 15.9 min, respectively (Figure 3.1).

Rapid HPLC analysis is commonly accomplished by increasing the mobile phase flow rate. Since analysis time is inversely related to flow rate, rapid equilibration between analytes and the stationary phase allows the reduction of the analysis time almost by half when the flow rate is doubled. However, flow rate is also proportional to the increase in column backpressure. In an attempt to enable faster HPLC analysis time without compromising the pressure drop across the column, we employed a silica monolithic column, the CSR (Table 3.1), to separate a mixture of CL. The gradient elution was optimized at a flow rate of 2.0 $mL \cdot min^{-1}$ (Table 3.2). Under these chromatographic conditions, the greater porosity of CSR enabled an increase in flow rate from 0.5 $mL \cdot min^{-1}$ on ZEX column to 2.0 $mL \cdot min^{-1}$ without any significant changes in backpressure. Moreover, the column length is proportional to analyte t_R, thus reduced column length results in the reduction of analysis time from 30 to 6 min. Compounds **3**, **4**, 1-Abu-CLB, and **2** eluted at 2.79, 3.23, 3.74, and 3.95 min, respectively, from the CSR column (Figure 3.2A). It was observed that changes in flow rate and gradient elution lead to a decline in the selectivity and more prominently, resolution of each CL (Table 3.3). On the other hand, capacity factor of individual CLs significantly improved when the CSR column was compared to the ZEX column. In spite of the drawbacks mentioned above, CSR column can still be considered desirable as it effectively resolved components of the CL mixture in shorter run times, which is required for high throughput analysis.

Table 3.2 Optimized solvent gradients for the chromatography columns using water (solvent A) and acetonitrile (solvent B)

ZORBAX Eclipse XDB-C_{18}™		**Chromolith® SpeedROD**		**Chromolith® Performance**		**Chromolith® High Resolution**		**POROS R1/20**	
Time (min)	Solvent B (%)	Time (min)	Solvent B (%)	Time (min)	Solvent B (%)	Time (min)	Solvent B (%)	Time (min)	Solvent B (%)
0	55	0	30	0	60	0	60	0	35
6.0	55	4.0	70	3.0	90	3.0	90	5.0	45
24.0	90	4.5	90	3.25	60	3.25	60	5.5	90
25.0	55	5.0	30	4.0	60	4.0	60	6.0	35
30.0	55	6.0	30					7.0	35

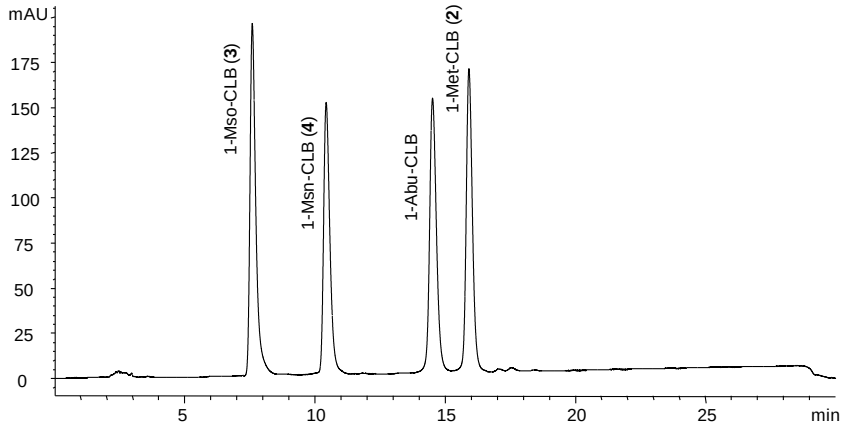

Figure 3.1 Chromatogram of a mixture of CLs and internal standard 1-Abu-CLB on the ZEX column at a flow rate of 0.5 mL·min^{-1}. Solvent program is as listed in Table 3.2.

Table 3.3 Chromatographic data of CL on different stationary phases under optimized elution gradients

Analyte	R	α	t_R (min)	RSD (%) t_R	k′	$W_{1/2}$	Symmetry	Stationary phase
1-Mso-CLB (**3**)			2.03	0.17	2.04	0.24	0.60	
1-Msn-CLB (**4**)	7.2	1.56	10.42	0.10	3.17	0.23	0.64	ZORBAX Eclipse XDB-C_{18}™
1-Abu-CLB	9.96	1.52	14.51	0.14	4.81	0.26	0.80	
1-Met-CLB (**2**)	3.33	1.12	15.89	0.17	5.36	0.25	0.78	
1-Mso-CLB (**3**)			2.79	0.02	5.20	0.06	0.57	
1-Msn-CLB (**4**)	4.09	1.19	3.23	0.03	6.17	0.06	0.52	Chromolith® SpeedRod
1-Abu-CLB	4.68	1.18	3.74	0.07	7.31	0.07	0.75	
1-Met-CLB (**2**)	1.77	1.06	3.95	0.07	7.78	0.07	0.61	
1-Mso-CLB (**3**)			1.05	0.04	0.52	0.03	0.63	
1-Msn-CLB (**4**)	3.55	1.64	1.28	0.04	0.85	0.04	0.68	Chromolith® Performance
1-Abu-CLB	5.96	1.71	1.70	0.07	1.46	0.04	0.79	
1-Met-CLB (**2**)	2.13	1.15	1.85	0.07	1.67	0.04	0.78	
1-Mso-CLB (**3**)			1.06	0.15	1.06	0.03	0.63	
1-Msn-CLB (**4**)	4.52	1.68	1.29	0.16	1.29	0.03	0.77	Chromolith ® High Resolution
1-Abu-CLB	8.06	1.72	1.70	0.14	1.70	0.03	0.83	
1-Met-CLB (**2**)	2.95	1.15	1.85	0.12	1.85	0.03	0.84	
1-Mso-CLB (**3**)			3.41	0.47	1.71	0.21	0.82	
1-Msn-CLB (**4**)	2.5	1.84	3.41	0.53	4.33	0.23	1.02	POROS R1/20
1-Abu-CLB	1.95	1.37	2.91	0.45	3.54	0.23	0.96	
1-Met-CLB (**2**)	2.41	1.36	4.39	0.22	5.86	0.25	1.00	

n = 3.

R: peak resolution, α: selectivity, t_R: retention time; RSD (repeatability): relative standard deviation; k′: capacity factor; $W_{1/2}$: peak width at half height

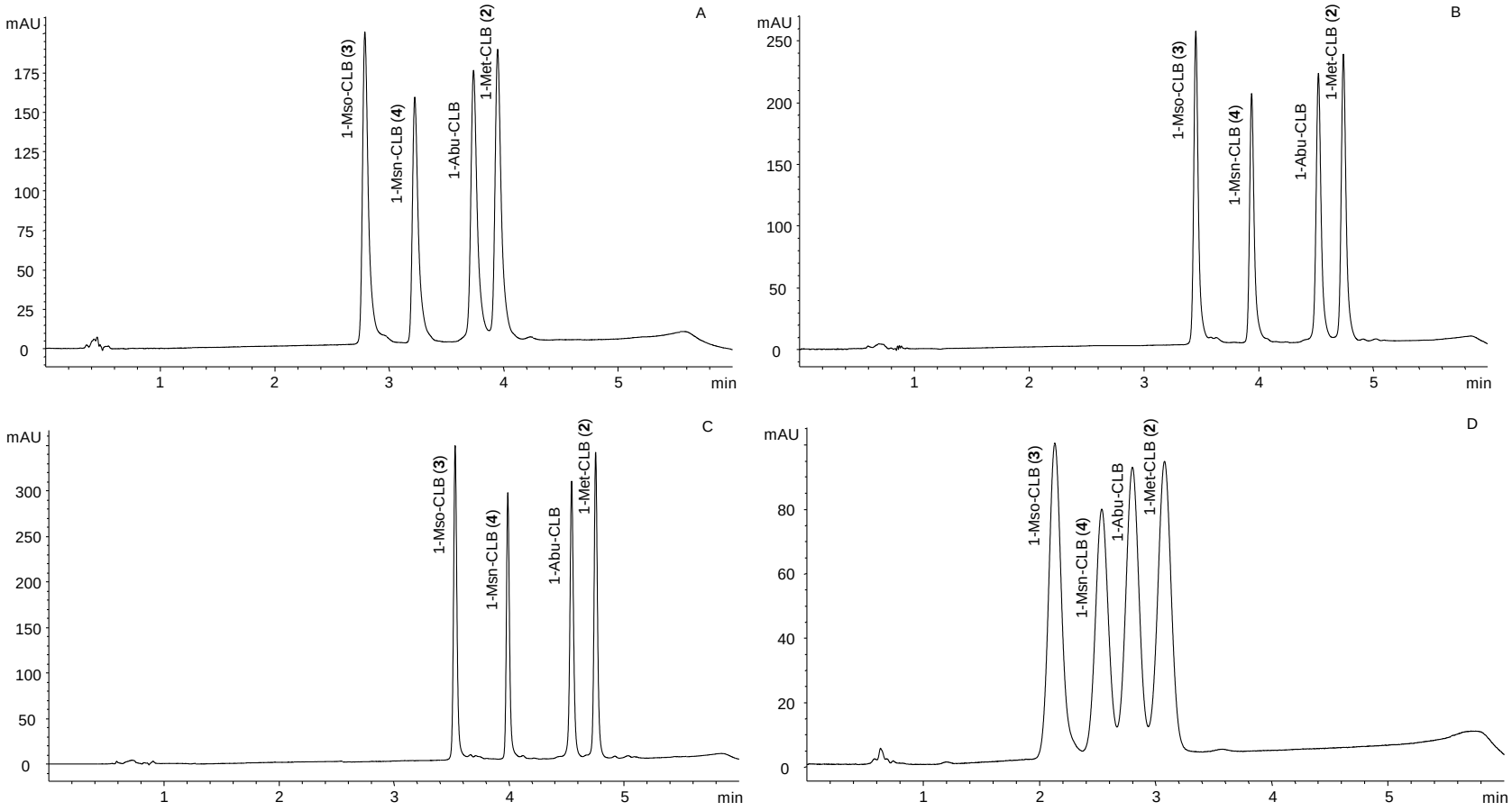

Figure 3.2 Chromatograms of a mixture of CLs and 1-Abu-CLB obtained using different columns: (A) Chromolith® SpeedROD, (B) Chromolith® Performance, (C) Chromolith® High Resolution, and (D) POROS. Gradient elution is as listed under SpeedROD in Table 3.2 at a flow rate of 2.0 $mL \cdot min^{-1}$ for all chromatographic separations

The performance of the CSR, CP and CHR and a particle-packed column, POR were subsequently compared. CP has double the column length (100 mm) of the CSR (50 mm). When applying an identical gradient elution previously used on CSR, the CP showed 1.4 times increase in resolution with no significant differences in selectivity (Table 3.4). As expected, t_R of **3**, **4**, 1-Abu-CLB, and **2** increased to 3.46, 3.95, 4.53, and 4.74 min, respectively. The retention time increase was accompanied by a reduction in band broadening (Figure 3.2B). On the other hand, CHR provided improvements in both speed and resolution with respect to the other two monolith columns. With the same gradient, resolution of individual CL was 1.5–2 fold higher than that observed with the CSR. No significant changes in selectivity and retention times were noted (Figure 3.2C).

When the same gradient elution was applied to the perfusion column (POR), both resolution and capacity factor decreased by half with respect to the CSR (Table 3.4). Band broadening (2-fold) was also observed across all CL peaks (Figure 3.2D). Consequently, individual CL from the mixture produced peaks without baseline resolution between the peaks. Surprisingly, the retention times of individual CL on the perfusion column were significantly shorter than observed on monolith columns. This suggests that, although both columns have the same dimension, the perfusion column contains a greater porosity than the monolith columns.

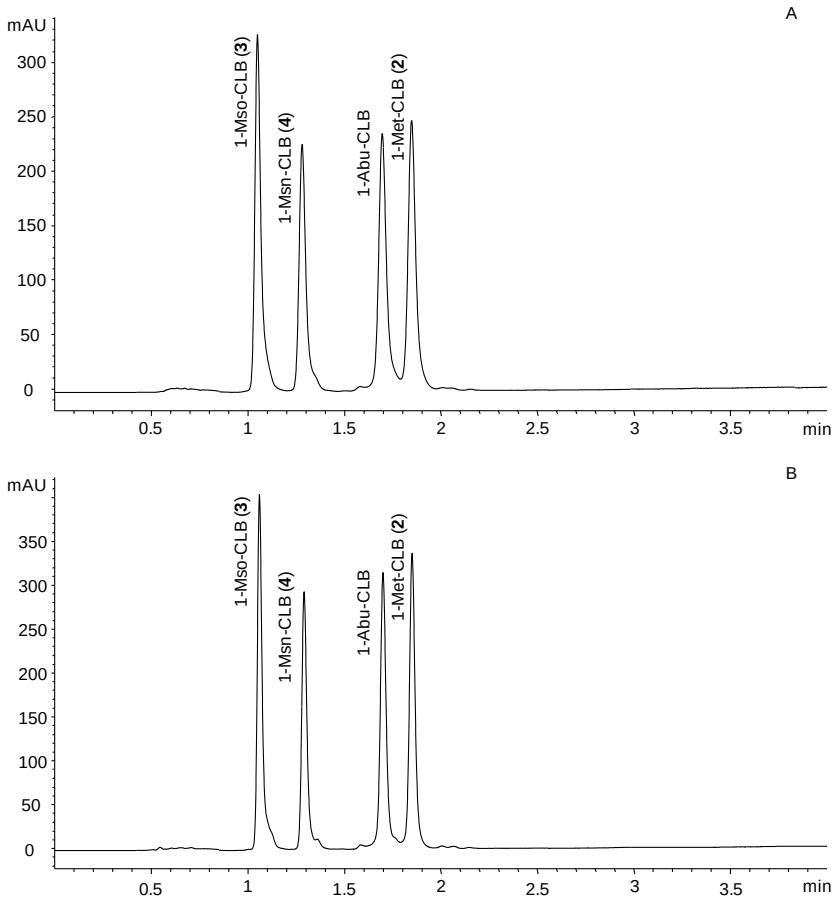

Figure 3.3 Chromatograms of a mixture of CLs and 1-Abu-CLB obtained from different columns: (A) Chromolith® Performance and (B) Chromolith® High Resolution. Optimized gradient elution is as listed under Performance/High Resolution in Table 3.2 at flow rate of 2.0 $mL \cdot min^{-1}$ for all chromatographic separations.

Table 3.4 Chromatographic data of CL on different stationary phases under elution gradient of that of Chromolith® SpeedROD

Analyte	R	α	t_R (min)	RSD (%) t_R	k′	$W_{1/2}$	Symmetry	Stationary phase
1-Mso-CLB (**3**)			2.79	0.02	5.20	0.06	0.57	Chromolith® SpeedRod
1-Msn-CLB (**4**)	4.09	1.19	3.23	0.03	6.17	0.06	0.52	
1-Abu-CLB	4.68	1.18	3.74	0.07	7.31	0.07	0.75	
1-Met-CLB (**2**)	1.77	1.06	3.95	0.07	7.78	0.07	0.61	
1-Mso-CLB (**3**)			3.46	0.17	4.01	0.05	0.82	Chromolith® Performance
1-Msn-CLB (**4**)	5.87	1.17	3.95	0.15	4.70	0.05	0.65	
1-Abu-CLB	6.63	1.18	4.53	0.13	5.54	0.05	0.92	
1-Met-CLB (**2**)	2.48	1.06	4.74	0.11	5.86	0.05	0.75	
1-Mso-CLB (**3**)			3.53	0.06	3.58	0.03	0.90	Chromolith ® High Resolution
1-Msn-CLB (**4**)	8.68	1.16	4.00	0.09	4.42	0.03	0.76	
1-Abu-CLB	9.44	1.17	4.55	0.15	4.99	0.04	1.13	
1-Met-CLB (**2**)	3.56	1.05	4.76	0.18	5.18	0.03	0.88	
1-Mso-CLB (**3**)			2.14	0.18	2.33	0.12	0.87	POROS R1/20
1-Msn-CLB (**4**)	2.00	1.28	2.80	0.02	2.97	0.13	0.98	
1-Abu-CLB	1.25	1.14	2.62	0.11	3.37	0.12	0.97	
1-Met-CLB (**2**)	1.26	1.13	3.08	0.10	3.80	0.13	0.97	

n = 3.

R: peak resolution, α: selectivity, t_R: retention time; RSD (repeatability): relative standard deviation; k′: capacity factor; $W_{1/2}$: peak width at half height

In order to develop a rapid screening method for flaxseed extracts, an improved HPLC gradient was developed for each column. These changes were then optimized for the CP, CHR, and POR (Table 3.2). Optimization focused on reduction of runtimes while maintaining or improving chromatographic resolution. The analysis time for CP and CHR were reduced from 6 min to 4 min from the initial CSR gradient. As shown in Figure 3.3, each CL eluted at similar times on both Chromolith® columns. Resolution of 1-Abu-CLB on CHR increased 1.7-fold, while peak width of all CLs declined 2-fold in comparison to the CSR column (Table 3.3). Meanwhile, selectivity of individual peptides increased 1.5-fold for CP and CHR compared to CSR.

To meet the goal of reducing the run times on CSR and optimization on POR column, the gradient elution developed for CP and CHR (Table 3.2) was applied. These chromatographic conditions led to significant loss of resolution (Appendix C.1) on the CSR column. On the other hand, a single broad fused peak visible on the HPLC-DAD chromatogram using the POR column signified that this elution gradient was not useful i.e. no separation of CL mixtures was achieved. Further modification of this solvent gradient by decreasing the organic solvent concentration coupled with the maintenance of 2.0 $mL \cdot min^{-1}$ flow rate led to separation of CLs in a 7 min run using the POR column, albeit with poor resolution (Appendix C.2). These observations suggested that gradient elution on POR column is not the best option for rapid HPLC analysis of CL mixtures.

3.4.2 Study of aqueous methanolic flax seed extract using monolithic and microparticulate columns

The optimized method for each of the five columns (Table 3.2) was tested for applicability in profiling of CLs occurring in aqueous methanolic extracts of flaxseed varieties. Such extracts comprise CLs **1**, **3**, **6**, **9**, **12**, and **15**. From these analyses, we observed that ZEX and CSR columns could separate the crude peptide mixture to relatively well-resolved peaks under 30 and 6 min, respectively (Figures 3.4A and B). On the other hand, **12** and **15** were not retained and eluted at void volume in CP, CHR and POR, indicating the poor suitability of the gradients for this application (Appendix C.3).

Further modifications on gradient elution for CSR and CHR were done in order to suit their applicability in rapid screening of flaxseed CLs in crude extracts. As a result, the total HPLC analysis time on CHR from 4.0 to 2.5 min was achieved with improved resolution of all peptide peaks (Appendix C.4). The CSR, with half of the column length of the CHR at a flow rate of 3.0 $mL \cdot min^{-1}$ and 28 ºC achieved similar resolution in 1.5 min (Figure 3.5), making it the better choice in rapid screening of flaxseed CLs.

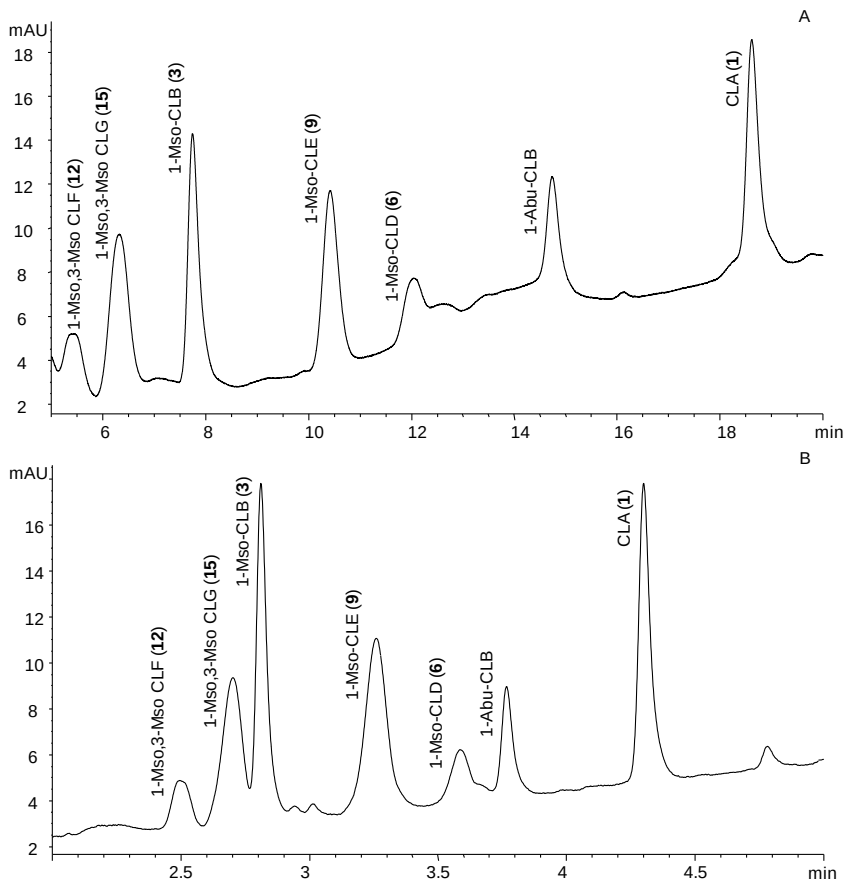

Figure 3.4 Chromatograms of methanol extract of flax seed showing mixture of 1-Mso,3-Mso-CLF (**12**), 1-Mso,3-Mso-CLG (**15**), 1-Mso-CLB (**3**), 1-Mso-CLE (**9**), 1-Mso-CLD (**6**), and CLA (**1**) with internal standard, 1-Abu-CLB obtained using different columns: (A) ZORBAX Eclipse XDB-C_{18} and (B) Chromolith® SpeedROD. Gradient elution is as listed in Table 3.2.

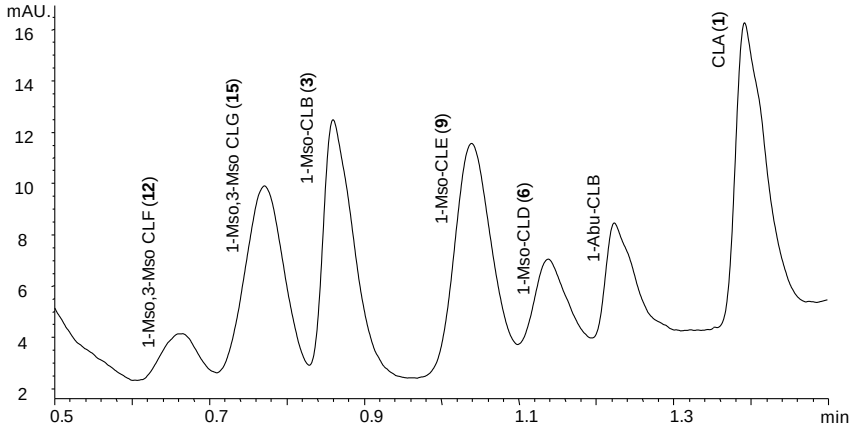

Figure 3.5 Chromatograms of methanol extract of flax seed showing mixture of 1-Mso,3-Mso-CLF (**12**), 1-Mso,3-Mso-CLG (**15**), 1-Mso-CLB (**3**), 1-Mso-CLE (**9**), 1-Mso-CLD (**6**), and CLA (**1**) with internal standard, 1-Abu-CLB obtained using Chromolith® SpeedROD at a flow rate of 3.0 $mL \cdot min^{-1}$ with the following gradient elution: 0 min 45% B, 1.0 min 80% B, 1.1 min 90% B, 1.2 min 45% B, 1.5 min 45% B.

3.4.3 Longevity of Chromolith® SpeedROD column

The longevity of CSR column was studied in a high-throughput screening of aqueous methanolic CL extracts of flax varieties. As mentioned previously, CSR column was chosen out of the five columns due to its excellent performance in separating CLs of flaxseed extracts within a shorter runtime as compared to ZEX column. Column longevity was evaluated by comparing HPLC parameters for 2000 injections of flaxseed extracts. Periodically the mixture of CL standards used in method development (Table 3.5) was injected to determine column performance. An increase in resolution, peak broadening, capacity factor, column efficiency, and back pressure were observed after the first 500 injections. However, no significant statistical variations in chromatographic parameters were observed, making monolithic column a suitable HPLC column for high throughput screening of CL.

Table 3.5 Chromatographic data of CL on Chromolith® SpeedROD column over multiple injections under optimized elution gradient

Analyte	R	α	t_R (min)	k′	$W_{1/2}$	Symmetry	Back-pressure	Injection
1-Mso-CLB (**3**)			2.79	5.20	0.06	0.62	71.5	0
1-Msn-CLB (**4**)	4.83	1.19	3.24	6.20	0.05	0.65		
1-Abu-CLB	5.47	1.18	3.75	7.33	0.06	0.77		
1-Met-CLB (**2**)	2.16	1.07	3.97	7.82	0.06	0.72		
1-Mso-CLB (**3**)			2.80	5.22	0.05	0.58	71.7	500
1-Msn-CLB (**4**)	5.31	1.19	3.25	6.22	0.05	0.58		
1-Abu-CLB	6.14	1.19	3.77	7.38	0.05	0.72		
1-Met-CLB (**2**)	2.48	1.06	3.98	7.84	0.05	0.66		
1-Mso-CLB (**3**)			2.80	5.22	0.05	0.57	74.8	1000
1-Msn-CLB (**4**)	5.19	1.19	3.24	6.20	0.05	0.58		
1-Abu-CLB	6.14	1.19	3.76	7.36	0.05	0.72		
1-Met-CLB (**2**)	2.48	1.07	3.97	7.82	0.05	0.67		
1-Mso-CLB (**3**)			2.81	5.24	0.05	0.57	78.3	1500
1-Msn-CLB (**4**)	5.19	1.19	3.25	6.22	0.05	0.56		
1-Abu-CLB	6.14	1.19	3.77	7.38	0.05	0.69		
1-Met-CLB (**2**)	2.48	1.06	3.98	7.84	0.05	0.64		
1-Mso-CLB (**3**)			2.81	5.24	0.05	0.58	78.3	2000
1-Msn-CLB (**4**)	5.19	1.19	3.25	6.22	0.05	0.58		
1-Abu-CLB	6.14	1.19	3.77	7.38	0.05	0.71		
1-Met-CLB (**2**)	2.48	1.06	3.98	7.84	0.05	0.66		

n = 3.

R: peak resolution, α: selectivity, t_R: retention time; k′: capacity factor; $W_{1/2}$: peak width at half height

3.5 Conclusion

The chromatographic performance of a conventional reversed phase HPLC column was compared to reversed phase monolithic and perfusion columns to evaluate their capability for separating a CL mixture and for high-throughput HPLC analysis. An optimized gradient was developed for each of the columns tested. The monolithic columns performed better than the perfusion column in these analyses. Shorter and rapid analyses were achieved with CP and CHR compared to other columns. We further established that increasing temperature from 23 ºC to 28 ºC at a flow rate of 3.0 $mL \cdot min^{-1}$ significantly increased the performance of CSR by allowing for shortening of analysis time to just 1.5 min without compromising the resolution. Longevity of monolithic column CSR was suitable for high-throughput screening as chromatographic parameters were constant over thousands of injections. The monolithic columns are suitable for high throughput screening of CL.

3.6 Connection to the next study

Monolithic CSR column was chosen to assist HPLC analysis for the next two studies. Performance and longevity of CSR column were deemed suitable for high throughput screening of CL from the world flax core collection.

CHAPTER 4
GENOTYPIC AND ENVIRONMENTAL VARIATION OF FLAX (*Linum usitatissimum* L.) CYCLOLINOPEPTIDES

4.1 Abstract

The flax (*Linum usitatissimum* L.) core collection is a representative subsample of the widest variation of flax gene resources. The collection, grown in Saskatoon, SK and Morden, MB in 2009, was screened using reversed phase HPLC to determine the concentration and composition of flaxseed cyclolinopeptides (CLs). The screening was optimized for high throughput analysis in a 96–well format. Optimum conditions for the extraction included grinding of flaxseed with ceramic beads, followed by incubation (60°C), separation of the solids from supernatant, oxidation of the filtered extract with hydrogen peroxide (H_2O_2, 4.5% v/v) for 1 h, and quenching of the oxidant with sodium thiosulfate ($Na_2S_2O_3$, 0.2 M). The concentrations of peptides encoded by NCBI gene #AFSQ01016651.1 were higher in most lines compared to those encoded by gene AFSQ01025165.1. Total CL content (milli-absorbance units, mAU) from core collection planted in Saskatoon ranged from 71.4 to 223.7 mAU with mean value ± standard deviation (SD) of 153.5 ± 24.3 mAU, while the CLs content from the Morden field ranged from 96.2 to 243.6 mAU with mean value ± SD of 171.3 ± 24.0 mAU. Among the accessions of the core collection, the highest levels of total CL were observed in the accession 'AC Watson', while, 'Primus' and an unnamed accession (CN 101580, TMP 10121) exhibited the highest ratio of CLs #AFSQ01016651.1 to CLs AFSQ01025165.1. Conversely, the lowest level of total CL and ratio of CLs #AFSQ01016651.1 to AFSQ01025165.1 was observed in accessions 'Hollandia' and 'Z 11637'. Potential relationship between CLs expression and plant morphological and chemical traits were investigated. No significant correlation was found between CL content and plant height, stem branching, petal colour, seed colour, seed weight, seed oil content, and α-linolenic (ALA) content.

4.2 Introduction

Domestication of flax (*Linum usitatissimum* L.) for the purpose of fibre and oil dates back to 10,000 BC (Zohary and Hopf, 2000). The fibre and oilseed types are commonly identified by their phenotypic characteristics, including height, stem branching, petal and seed colour. PGRC has assembled more than 3,000 flax lines from 72 countries, representing historic or present flax cultivation regions (Diederichsen and Fu, 2008) and selected 380 accessions, dubbed as flax core collection. The term core collection has been linked to a collection representing 'minimum repetitiveness, the genetic diversity of a crop species and its relatives' (Frankel, 1984). 'CDC Bethune', 'Macbeth' and 'Hanley' were included as checks. The accessions were described as medium-late maturing oilseed flax with moderate resistancy to wilt caused by *Fusarium oxysporum* f. sp. *lini* and immunity to rust by *Melampsora lini* (Rowland *et al*., 2002; Duguid *et al*., 2003a; b). The core collection and checks were regenerated in summer 2009 in Saskatoon, SK (seedling and harvesting on May 26th and Oct 14th, respectively) and Morden, MB (seedling and harvesting on June 2nd and Oct 13th, respectively). The soil of Saskatoon was described as loamy, dark brown chernozemic, while Morden as clay loam, black chernozemic, (Diederichsen *et al*., 2006).

Knowledge about the chemical composition of flax varieties is crucially important in order to understand genetic variation and potentially develop value-added uses for flax. Emerging knowledge in regard to the compounds in flax that impart health properties will lead to the search for these compounds within flax germplasm collections and possibly breeding effort to enhance these compounds (Diederichsen and Fu, 2008). The content of mucilage, stem fibre and oil have been assessed for many flax germplasm accessions (Diederichsen, *et al*., 2006; Diederichsen and Ulrich, 2009; Bjelková, *et al*., 2012). To date, there are no published studies reporting the content and type of cyclic peptides present in flax. Cyclolinopeptides (CLs), the cyclic peptides from flax are comprised of 8 to 9 amino acid residues, with molecular weights approximately 1 kDa (Table 2.2 and Figure 2.5). The NCBI gene sequence accession #AFSQ01016651.1 embedded sequences of **1** (ILVPPFFLI), **2** (MLIPPFFVI), and **8** (MLVFPLFVI), whereas NCBI gene AFSQ01025165.1 embedded sequences of **5** (MLLPFFWI), **11** (MLMPFFWV) and **14** (MLMPFFWI) (Sayers *et al*., 2009). Biological activities, including immunosuppressive (Wieczorek, *et al.,* 1991), antimalarial (Bell *et al*., 2000), and cytoprotective (Kessler *et al.,* 1986) have been observed from *in vitro* and *in vivo* studies involving CLs. In the

current study, the relationships between CL content and flax morphological and chemical traits were investigated. Morphological traits of flax were accessed through PGRC website (http://pgrc3.agr.gc.ca/acc/search-recherche_e.html), evaluation approach of the traits was described by Diederichsen and Ulrich (2009). The traits included in this study are plant height, stem branching, petal colour, seed colour, seed weight, seed oil content, and ALA content. .

The present study aimed to: (i) investigate genetic variation on profile and comparative level of CLs from flax accessions in the core collection grown in Saskatoon, SK and Morden, MB in 2009 and (ii) consider the CLs content in relation to morphological traits of plant. High-throughput screening of CLs in flaxseed varieties was conducted by employing 96–well plate format during extraction and HPLC analysis of CLs. The outcome of this study is an insight into the chemical diversity of flax that benefits breeding programs in improving the chemical composition and functional properties of cultivated flax.

4.3 Experimental

4.3.1 Reagents and samples

Preliminary experiments to test the effects of sample size, temperature, and grinding on the yield of CLs were conducted on flax 'CDC Bethune', grown in Saskatoon, SK in 2008. The CL profile was studied for samples obtained from the PGRC core collection regenerated in Saskatoon, SK and Morden, MB in 2009. All seed material was kindly donated by Dr. Scott Duguid (Morden Research Station, MB). Analysis was conducted on duplicates. Water (H_2O) was purified to 18.2 MΩ cm on a Milli-Q Integral system (Millipore, Molsheim, France). Chemicals used in the conduct of this study are listed in Appendix A. Preparation and purification of standard 1-Abu-CLB was described in section *3.3.1*. Quality report of the standard is presented in Appendix B.

4.3.2 Sample preparation

4.3.2.1 Effect of sample size, temperature, and grinding on the yield of cyclolinopeptides

Preliminary experiments concerning the effect of sample size, temperature, and grinding on the yield of flaxseed peptides were conducted in eppendorf tubes (Fischer Scientific, USA). Three sets of flaxseed: 8 (0.0460 g), 25 (0.1422 g), and 200 seeds (1.1988 g) were prepared. The seeds were ground (Mixer Mill MM 300, F. Kurt Retsch GmbH & Co. KG, Haan, Germany) at

25 s^{-1} for 10 min in the presence of 3 metal beads. Ground flaxseed was incubated with 70% aq. MeOH in H_2O (1:10, w/v) for 2 h at 60 °C (Bio/CAN Scientific, Mississauga, Ontario, Canada). Before and after incubation, the mixture was mixed using a vortex mixer (Vortex-Genie 2 Model G-560, 12-812, Fisher Scientific, NY, USA). The mixture was centrifuged at 10.612 × g for 10 min (Eppendorf centrifuge 5417C, Eppendorf AG, Hamburg, Germany). The supernatant was filtered with 0.45 µM PTFE syringe filter (Whatman Ltd., Psicataway, NJ) and analyzed by HPLC. The effect of grinding and incubation temperature on the extraction efficiency of peptide was investigated simultaneously. Following degumming, flaxseeds were treated with or without grinding. The samples were then extracted with 70% aq. MeOH (1:10, w/v) and incubated for 2 h at either r.t. or 60 ºC. The samples were centrifuged, filtered, and extracts were analyzed by HPLC.

4.3.2.2 Effect of oxidation and quenching on the stability of cyclolinopeptides

Oxidation of CLs using H_2O_2, sodium periodate ($NaIO_4$) and potassium iodate (KIO_3) was investigated by direct addition of oxidants to the flaxseed extract. Varying concentrations (1.5, 3, 4.5, 6, and 12%) of oxidants were evaluated to determine the concentration required for optimum peptide oxidation. Sodium metabisulphite ($NaHSO_3$) and $Na_2S_2O_3$ were evaluated as possible quenching agent for CLs oxidation. The trial involved addition of 0.2 M $NaHSO_3$ in aq. MeOH in H_2O (50%, v/v) or 0.2 M $Na_2S_2O_3$ in aq. MeOH in H_2O (70%, v/v), in various ratios to H_2O_2 (1:1, 0.5:1, 1.5:1, and 2:1, respectively) to flaxseed extract.

4.3.2.3 Cyclolinopeptides profile of the flax core collection

Approximately 120 mg of flaxseed was weighed and placed into a 96–well plate, 2.2 mL (VWR, Radnor, PA). Pre-heated H_2O (60 °C, 1:10 w/v) was added to each well using a multichannel pipette (8-channel Biohit e1200, Finland). The plate was covered with a sealing mat (VWR, Radnor, PA) and incubated at 60 °C for 2 h (VWR model 1350GM, Cornelius, OR). The extracted gum was decanted. The remaining flaxseed was ground at 1400 strokes/minute for 10 min (2010 Geno/grinder®, SPEX CertiPrep, Inc., Methucen, NJ) with the help of spherical 5.0 mm Zirconia Grinding Media (Inframat Advanced Materials LLC, Manchester, CT). With a multichannel pipette, 100 µL of 1-Abu-CLB **(**0.1 mg·mL^{-1}**)** was added to the mixture as the internal standard for CLs. Aqueous 860 µL of MeOH in H_2O (78%, v/v) was added to the

mixture to make final dilution of 1:8, w/v. The mixture was mixed using Geno/Grinder for 2 mins at 1400 strokes/minute and subsequently incubated at 60°C for 2 h. Samples were centrifuged at 1760 × g for 20 min (Eppendorf centrifuge model 5804R, Habsburg, Germany) to settle the flaxseed solids. The supernatant (400 µL) was subsequently transferred to a filter plate (96–well, Pall Corporation, Ann Harbor, MI) to remove particulate matter. The filter plate was covered and placed on top of a receiving 96–well plate and the assembled plates were placed in the centrifuge to facilitate filtration (1760 × g for 10 min). The filtered methanol extract (100 µL) was transferred to a 96–well HPLC, 0.5 mL (Agilent Tech, Denmark). The extract was oxidized with 50 µL H_2O_2 (4.5%, v/v) in H_2O at room temperature for 1 h. The reaction was quenched with 150 µL $Na_2S_2O_3$ (0.2 M) in 70% aq. MeOH in H_2O. The samples were covered with 96–well cap mat (Thermoscience, Rochester, NY) and injected directly for HPLC analysis.

4.3.3 HPLC apparatus

Details pertaining to the chromatographic separation of CLs are described in *section 3.3.2*. The solvent gradient is listed in *section 3.3.3* under Chromolith® SpeedROD. Eluting peaks were detected at wavelengths of 214 with a 10 nm bandwidth and 244 nm with a 20 nm bandwidth against a reference signal at 300 nm with a 10 nm bandwidth, and at 280 nm with a 16 nm bandwidth and against a reference signal at 360 nm with a 100 nm bandwidth, using Chemstation LC 3D™ system software (Agilent Technologies Canada Inc., Mississauga, ON). Area integration of eluting peak was recorded at 214 nm. Injection volume for CLs was maintained at 10 µL for all investigations.

4.3.4 Statistical data analysis

CL content was proportional to sum of UV absorbance (mAU) of the observed peptide chromatographic peak at 214 nm. T-test was used to evaluate the significances between mean total CL content. Relationship between total CL and traits (plant height, seed weight, seed oil content, and ALA content) were determined using Pearson correlation. Relations of stem branching, petal colour, and seed colour to the total CL content were evaluated by descriptive statistics (minimum, maximum, mean, standard deviation, coefficient of variation).

4.4 Results and discussion

4.4.1 Effect of sample size, temperature, and grinding on the yield of cyclolinopeptides

The limited amount of seeds of the flax core collection and limited volume capacity of 96–well plate required the extraction to be optimized for the least sample size. Extraction of flaxseeds yielded CLs mostly in their reduced forms i.e., **1**, **2**, **5**, **8**, **11**, and **14** (Table 2.2). The CL profile appeared similar, regardless of the number of seeds used (Figure 4.1A). The chromatograms of methanol extract of flaxseed indicated elevated CLs level by 2–3 fold as sample sizes increased from 8 to 25 seeds. There was no significant difference observed on peptides yield from 25 seeds and those from 200 seeds. In the sense of profiling purposes, CLs profile was not affected by sample sizes since the dilution factor of seeds and solvent was maintained (1:10 w/v). Hence, the consistency of CLs ratio between 25 and 200 seeds was expected. On the other hand, the significant difference of CLs ratio between 8 and 25 seeds gave evidence to pipetting error that occurred when solvent level was too low. Incubation of ground flaxseed at 60 ºC appeared as the best treatment to extract CLs (Figure 4.1B). The level of CLs extracted from ground flaxseed is higher than that from whole flaxseed by 1.4 to 2 fold and 3.4 to 4.5 fold when followed by incubation at r.t. and 60 ºC., respectively. The result is expected due to the nature of CLs as hydrophobic compounds occurring in flax oil. Grinding provided mechanical disruption to the seed cell wall and therefore, released flax oil together with lipid soluble compounds. Subsequent addition of 70% aq. MeOH extracted the peptides and left behind the oil. The yield of CLs was more prominent when the seeds were incubated at elevated temperature. An increased level of CLs by 1.1 to 1.3 fold and 2.3 to 2.6 fold were observed when the ground and whole seeds were extracted at 60 ºC, respectively.

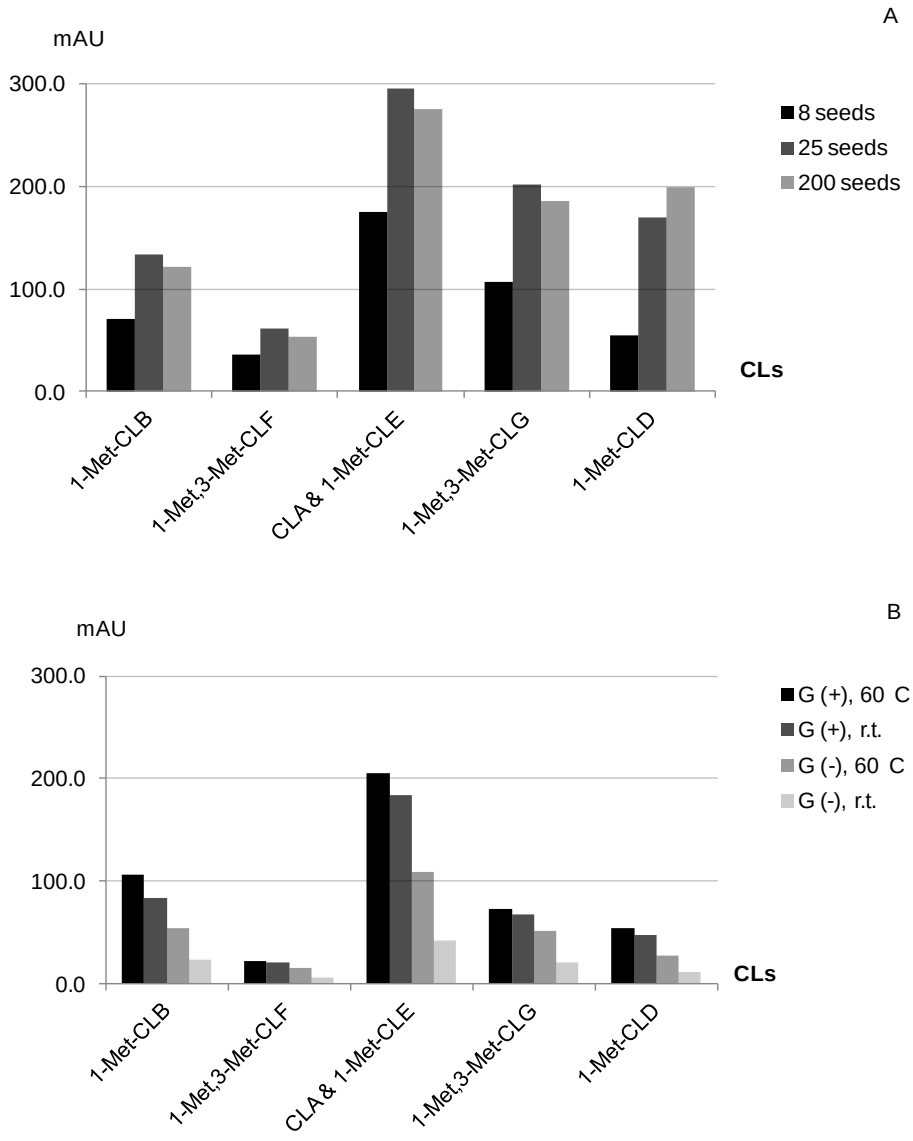

Figure 4.1 Effect of (A) sample size and (B) grinding and incubation temperature on the yield of cyclolinopeptides in the unoxidized extract of flaxseed

4.4.2 Effect of oxidation and quenching on the stability of cyclolinopeptides

The reduced forms of CLs are undesirable because they hindered CLs profiling. Chromatographic separation of unoxidized flaxseed showed co-elution of CLA with 1-Met-CLE. Total of six peptides i.e. **1**, **2**, **5**, **8**, **11**, and **14** eluted within 1 min period. Oxidation of Met residue to Mso was successfully achieved by direct addition of H_2O_2 to flaxseed methanol extract. Oxidation of Met to Mso converted **2**, **5**, **8**, **11**, and **14** to **3**, **6**, **9**, **12**, and **15**, respectively. The Mso-containing peptides eluted within 2 min on the same column and solvent gradient, with retention times of 2.5, 2.7, 2.8, 3.2, and 3.5 for **12**, **15**, **3**, **9**, and **6**, respectively (Figure 3.4B). Oxidation did not occur in samples with KIO_3. CLs in samples with $NaIO_4$ were oxidized, although loss of tryptophan-containing peptides that may be attributed to the decomposition of the peptides was observed. The oxidation was quenched in order to prevent over-oxidation of Mso to Msn. Over-oxidation of peptides was undesirable because of co-elution of **4** and **3**, as well as **10** and **6**. Uneven conversion of Mso to Msn hindered qualitative and quantitative evaluation of CLs. HPLC/ESI-MS/MS spectra of over-oxidized flaxseed extract showed the presence of **4**, **3**, and compounds with *m/z* at 1080.6, 1100.5, 1114.5, and 1130.5 (Figure 4.2). The compounds correspond to the molecular weight of known CLs after the addition of an oxygen (O) on CLs **6**, **12**, **15** and 2 O on **15**, respectively.

Both $NHSO_3$ and $Na_2S_2O_3$ quenched CLs oxidation. However, $Na_2S_2O_3$ was more suitable quenching agent because of its solubility in 70% aq. MeOH. Solvent effect on peptides precipitation was minimized when employing $Na_2S_2O_3$ than $NHSO_3$ which is insoluble in 70% aq. MeOH. The stability of mixture containing 100 µL methanol extract of flax seed, 50 µL H_2O_2 (4.5 %, v/v)) and 150 µL $Na_2S_2O_3$ (0.2 M) was monitored through HPLC over a period of 7 h HPLC analysis (Figure 4.3). The formula has taken into account complete conversion of Met to Mso, the maximum volume of individual well (300 µL with lid on), the maximum concentration of reagent before precipitation occurred, and the detectable signal of peptides.

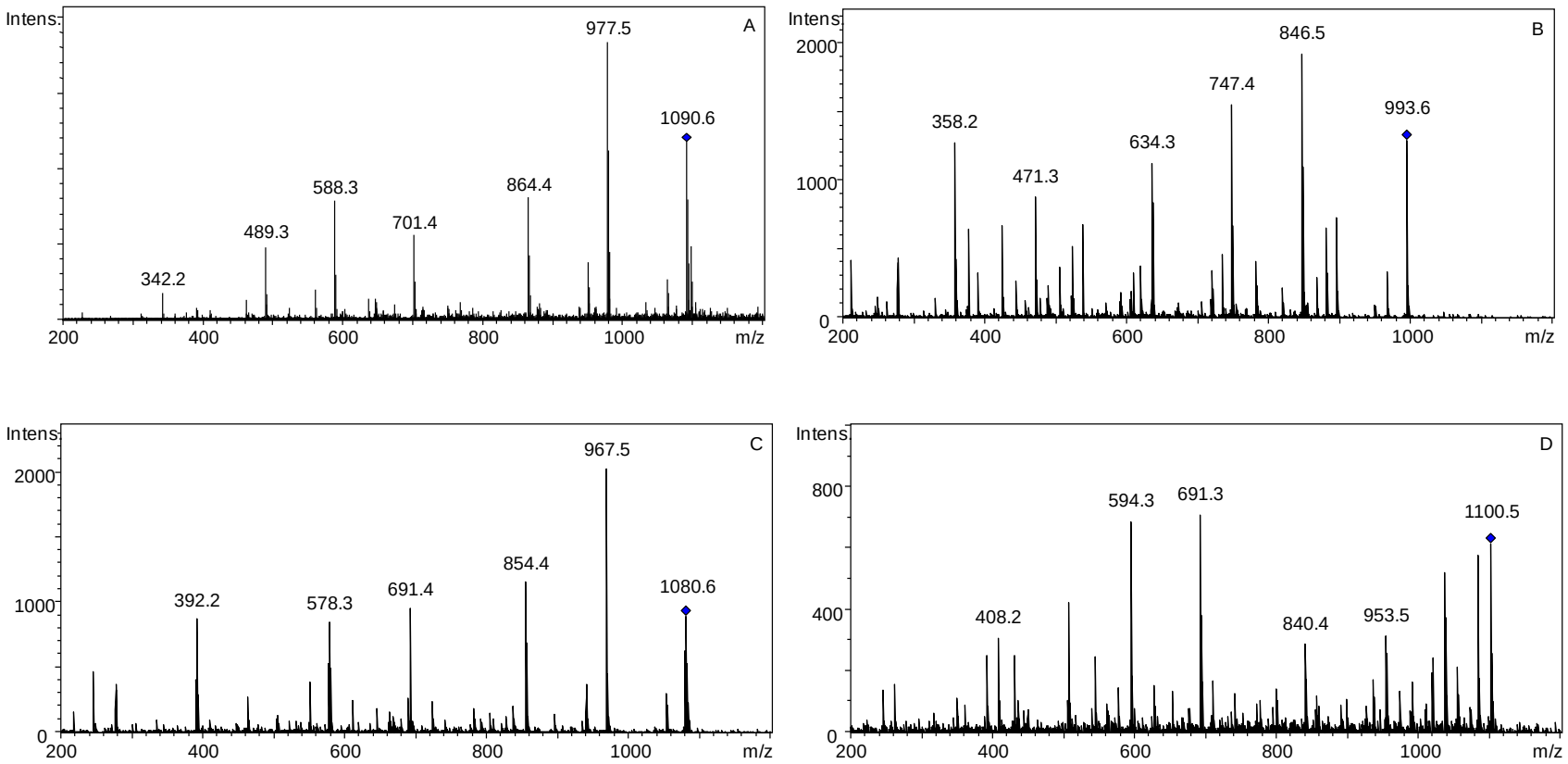

Figure 4.2 HPLC/ESI-MS/MS spectra of over-oxidized methanol extract of flaxseed showing (A) 1-Msn-CLB (**4**), (B) 1-Msn-CLE (**3**), and compounds with *m/z* at (C) 1080.6, (D) 1100.5, (E) 1114.5, and (F) 1130.5 (con't)

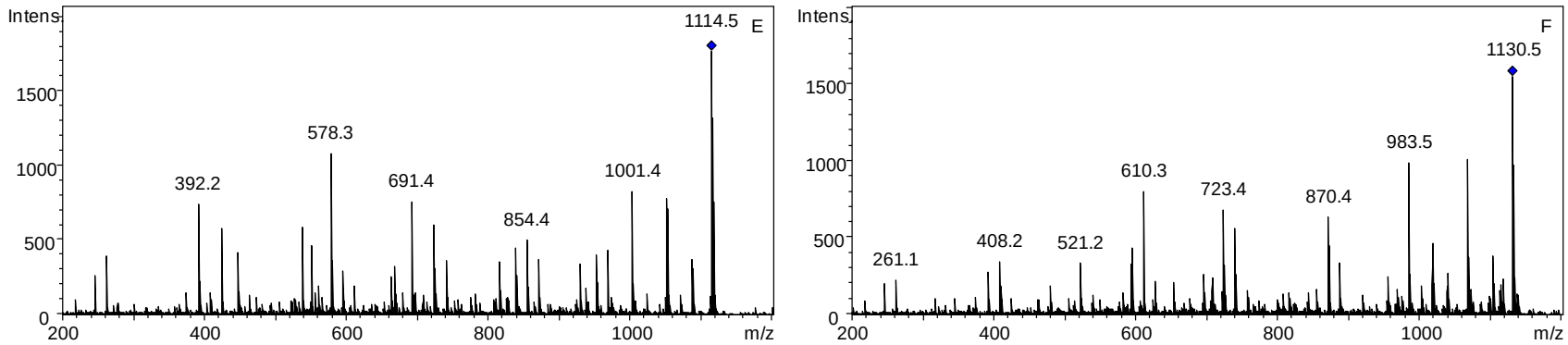

Figure 4.2 MS/MS spectra of over-oxidized methanol extract of flaxseed showing (A) CLK (**4**), (B) CLJ (**3**), and compounds with *m/z* at (C) 1080.6, (D) 1100.5, (E) 1114.5, and (F) 1130.5 (con't)

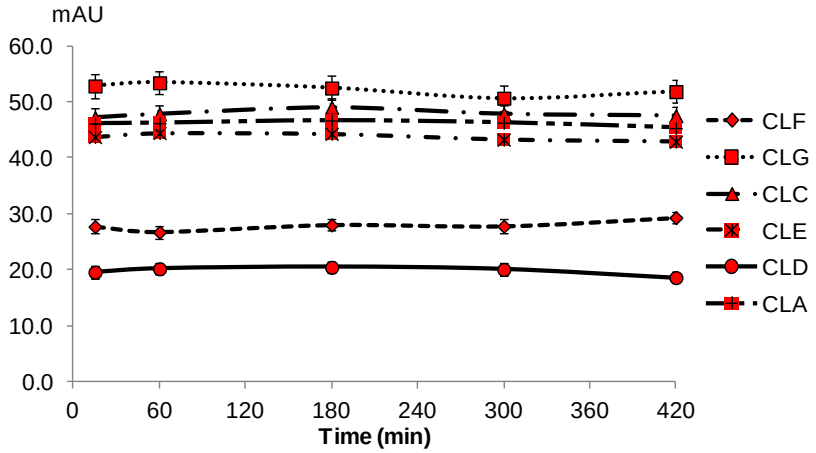

Figure 4.3 Distribution of CLs in a mixture of flaxseed extract (100 µL, 70% aq. MeOH v/v in H_2O), H_2O_2 5(50 µL, 4.5 % v/v), and $Na_2S_2O_3$ (150 µL, 0.2 M) detected over 7 hour after the addition of $Na_2S_2O_3$

4.4.3 Cyclolinopeptides profile of the flax core collection

Flax lines from the core collection planted in Saskatoon, SK and Morden, MB in 2009 were screened for CLs profile. The oxidized extracts of all accessions contain the previously known CLs **1, 3, 6, 9, 12,** and **15**. Profile of observed peptides contained in flax core collection planted in Saskatoon, SK and Morden, MB are shown in Appendix D (as mean value of two replications). In exception of two accessions ('Hollandia' and 'Z 11637'), the CLs profiles of flax accessions planted in both locations showed high similarity. Detection of CLs by diode array detector was due to chromophores peptide bonds, phenylalanine, and tryptophan that can absorb UV light and provide signal to the detector at 214, 260, and 280 nm, respectively (Pace *et al*., 1995; Marshak, 1996). CLs showed comparatively strong absorbances at 214 nm due to the peptide bonds and conjungated double bronds present in aromatic amino acids, hence integration area was determined based on eluting peaks at 214 nm. Comparison of three check accessions ('CDC Bethune', 'Hanley', and 'Macbeth') between the two locations showed the extraction consistency (Table 4.1). The repeated check 'CDC Bethune' was systematically positioned at every fifth plots. The correlations between peptides contained in the accessions of flax core collection was presented in Table 4.2. In exception of **6**, there were strong correlations between peptides encoded by the same gene. Correlation within the peptides encoded by gene AFSQ01016651.1 is higher than the peptides embedded in gene AFSQ01025165.1 (Table 4.3). CLs **1** to **3** and **9** were shown to have r value of 0.9497 and 0.8071, respectively. Correlation between **3** to **9** was weaker, with r value of 0.7843. Strong correlation within gene AFSQ01025165.1 can only be seen from **12** to **15** (r = 0.8718). Correlation between CLs **6** to **12** and **15** are lower than the rest with only r value of 0.3362 abd 0.2518, respectively.

Table 4.1 Distribution of CLs (mAU) in check accessions (CDC Bethune, Hanley, and Macbeth) of core collection grown in Saskatoon, SK and Morden, MB in 2009

Location	**Saskatoon, SK**			**Morden, MB**		
	CDC Bethune [a]	**Hanley [b]**	**Macbeth [b]**	**CDC Bethune [a]**	**Hanley [b]**	**Macbeth [c]**
1-Mso,3-Mso-CLF (12)	12.1 ± 1.7	12.0 ± 3.9	15.0 ± 3.2	14.8 ± 2.8	13.0 ± 0.9	15.6 ± 1.8
1-Mso, 3-Mso-CLG (15)	36.9 ± 5.8	36.8 ± 11.5	42.6 ± 6.8	41.3 ± 5.9	40.5 ± 3.4	46.3 ± 4.3
1-Mso-CLB (3)	38.7 ± 4.1	38.3 ± 8.8	41.4 ± 7.7	43.4 ± 4.9	41.7 ± 3.7	47.8 ± 2.6
1-Mso-CLE (9)	41.1 ± 5.1	42.7 ± 8.9	48.7 ± 7.5	46.8 ± 5.0	44.4 ± 1.8	53.6 ± 4.5
1-Mso-CLD (6)	15.3 ± 2.7	14.9 ± 17.0	4.2 ± 16	3.0 ± 2.1	16.1 ± 1.9	17.4 ± 1.9
CLA (1)	40.7 ± 4.2	39.6 ± 7.0	44.3 ± 4.2	44.6 ± 3.9	41.8 ± 1.1	48.1 ± 2.7
Total	184.8 ± 20.3	184.3 ± 40.1	209 ± 30.4	207.0 ± 17.9	197.5 ± 8.6	228.8 ± 10.8
Ratio	1.9 ± 0.2	2.0 ± 0.4	1.9 ± 0.1	1.9 ± 0.2	1.9 ± 0.1	1.9 ± 0.1

a N = 500 × 2

b N = 6 × 2

c N = 5 × 2

Table 4.2 Correlation coefficient of the levels of CLs encoded by genes AFSQ01016651.1 **(1, 3, 9)** and AFSQ01025165.1 **(6, 12, 15)** in the flaxseed accessions from the core collection (†P < 0.01)

N = 1000 (duplicate)	**CLA (1)**	**1-Mso-CLB (3)**	**1-Mso-CLD (6)**	**1-Mso-CLE (9)**	**1-Mso,3-Mso-CLF (12)**	**AFSQ01016651.1 (1, 3, 9)**
1-Mso-CLB (3)	0.95†					
1-Mso-CLD (6)	0.15	0.17				
1-Mso-CLE (9)	0.81†	0.78 †	0.19			
1-Mso,3-Mso-CLF (12)	0.31	0.32	0.34	0.39		
1-Mso,3-Mso-CLG (15)	0.28	0.31	0.25	0.33	0.87†	
AFSQ01025165.1 (6, 12, 15)						0.25

Table 4.3 Correlation coefficients of the levels of CLs encoded by genes AFSQ01016651.1 **(1, 3, 9)** and AFSQ01025165.1 **(6, 12, 15)** in the flaxseed accessions between the core collection planted in Saskatoon, SK and Morden in 2009 ($P < 0.01$)

CLs	**r**
CLA (1)	0.6478
1-Mso-CLB (3)	0.6037
1-Mso-CLD (6)	0.5536
1-Mso-CLE (9)	0.5359
1-Mso,3-Mso-CLF (12)	0.4707
1-Mso,3-Mso-CLG (15)	0.5221
AFSQ01025165.1 (6, 12, 15)	0.5165
AFSQ01016651.1 (1, 3, 9)	0.5813

The total CL content of samples from the core collection planted in Saskatoon ranged from 71.4 to 223.7 mAU with a mean value of 153.5 ± 24.3 mAU ($x \pm SD$), while the mAU from samples of the Morden site ranged from 96.2 to 243.6 mAU with mean value of 171.3 ± 24.0 mAU ($x \pm SD$). Accessions expressing the lowest and highest CLs levels were listed in Table 4.4. The variation of total CL in both locations resembled normal distribution (Figures 4.4A1 and 2), with about 82% of the values within 1SD away from the mean (μ). Accession 'AC Watson' (accession no CN 18973, TMP 605) was identified as the line expressing highest CL content in both locations. When compared to the mean value of the respective locations, total CL level in AC Watson reached more than 2SD greater than the mean of all accessions. On the other hand, accession 'Hollandia' (CN 98056, TMP 2181) and 'Z 11637' (CN 98150, TMP 2192) expressed the lowest level of CL. CL content of the collection planted in either location showed a positive ($r = 0.5548$) and significant ($P < 0.01$) correlation (Figure 4.5A).

Level of peptides coded by genes AFSQ01016651.1 (containing embedded sequences for CLs **1, 3, 9**) were compared to those coded by AFSQ01025165.1 (containing embedded sequences for CLs **6, 12, 15**). Majority of the accessions (95%) expressed higher cumulative level of CLs **1, 3,** and **9** than CLs **6, 12,** and **15**, showing higher expression of gene AFSQ01016651.1 than AFSQ01025165.1. Accessions 'Primus' (CN 98689, TMP 8216-5) and an unnamed accession (CN 101580, TMP 10121) expressed the highest ratios of CLs **1, 3, 9** in comparison to CLs **6, 12, 15** in both locations (Table 4.5). Lowest ratios of CLs **1, 3, 9** to CLs **6, 12, 15** were observed in accessions 'Hollandia' and 'Z 11637'. Approximately 79% of the accessions in Saskatoon were within 1SD away from the mean of all accessions (2.2 ± 0.5) as compared to 85% accessions in Morden (2.1 ± 0.5) (Figures 4.4B1 and 2). The correlation between gene expressions in the two locations was positively significant ($r = 0.5798$, $P < 0.01$) (Figure 4.5B).

Table 4.4 Flax accessions with lowest and highest total CL content (mAU)

TMP	CN	Accession name	Origin	Height (cm)	Stem branching	Seed weight (mg)	Seed colour	Petal colour	Total CL (mAU)	
									SK	MB
TMP-605	18973	'AC Watson'	Canada	51	1/4 branched	6.3	medium brown	blue	222.6	243.6
TMP-2181	98056	'Hollandia'	Netherlands	71	1/5 branched	4.2	medium brown	lavender	71.4	101.4
TMP-2192	98150	'Z 11637'	Netherlands	91	1/4 branched	3.8	medium brown	white	88.1	96.2

Table 4.5 Flax accessions with lowest and highest ratio of CLs encoded by AFSQ01016651.1 [CLA (**1**), 1-Mso-CLB **(3),** 1-Mso-CLE **(9)**] to CLs encoded by AFSQ01025165.1 [1-Mso-CLD **(6)**, 1-Mso,3-Mso-CLF **(12),** 1-Mso,3-Mso-CLG **(15)**

TMP	CN	Accession name	Origin	Height (cm)	Stem branching	Seed wt. (mg)	Seed colour	Petal colour	Ratio	
									SK	MB
TMP-10121	101580	_	Poland	47	1/2 branched	7.6	yellow	pink	3.7	3.5
TMP 8216-5	98689	'Primus'	Poland	45	1/3 branched	8.3	medium brown	lavender	4.1	3.7
TMP-2192	98150	'Z 11637'	Netherlands	91	1/4 branched	3.8	medium brown	white	0.6	0.7
TMP-2181	98056	'Hollandia'	Netherlands	71	1/5 branched	4.2	medium brown	lavender	0.7	0.7

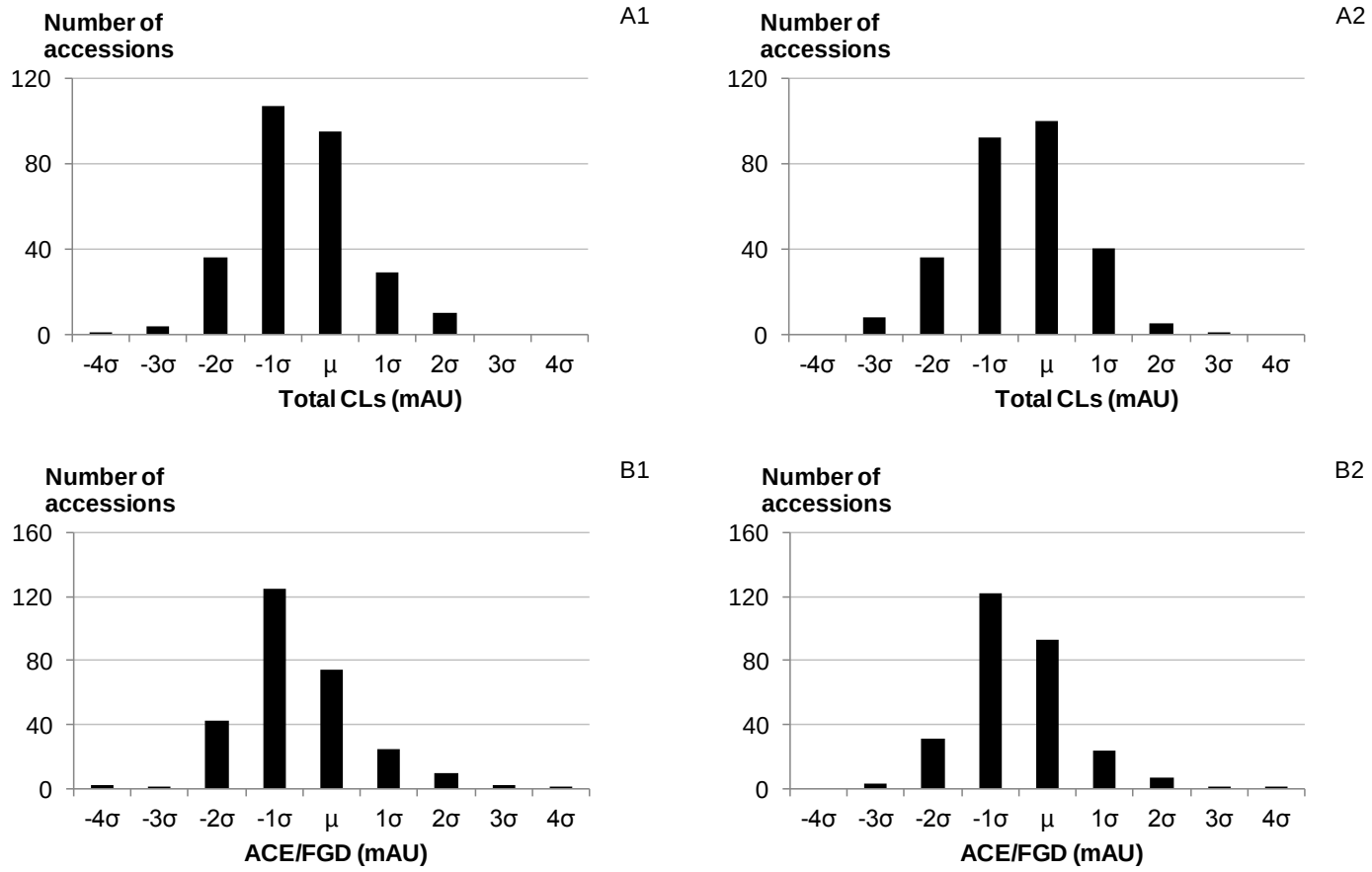

Figure 4.4 Histogram showing (A) total CL contents (mAU) and (B) comparison of encoded by AFSQ01016651.1 [CLA (**1**), 1-Mso-CLB **(3),** 1-Mso-CLE **(9)**] to CLs encoded by AFSQ01025165.1 [1-Mso-CLD **(6)**, 1-Mso,3-Mso-CLF **(12),** 1-Mso,3-Mso-CLG **(15)** expressed by 282 accessions of the core collection planted in (1) Saskatoon, SK and (2) Morden, MB in 2009. σ: standard deviation (SD)

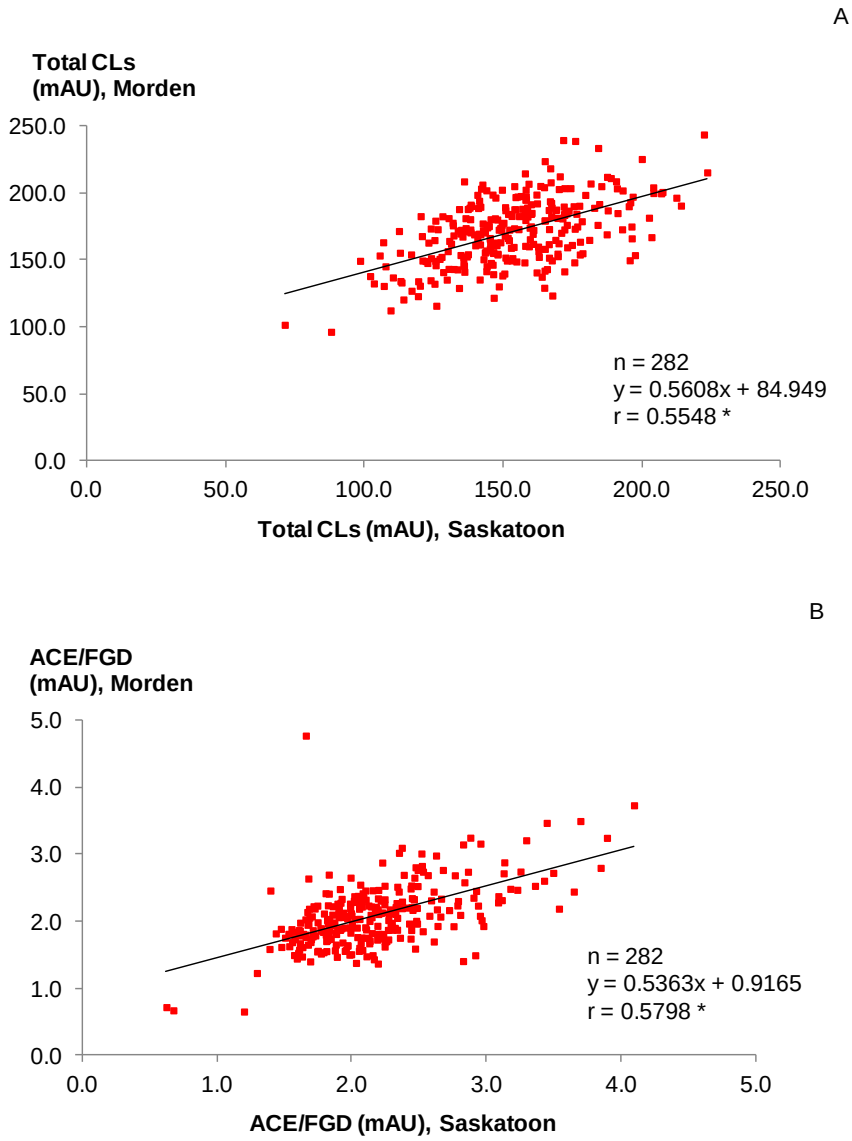

Figure 4.5 Correlation between (A) total CL content and (B) comparison of CLs encoded by AFSQ01016651.1 [CLA (**1**), 1-Mso-CLB **(3),** 1-Mso-CLE **(9)**] to CLs encoded by AFSQ01025165.1 [1-Mso-CLD **(6)**, 1-Mso,3-Mso-CLF **(12),** 1-Mso,3-Mso-CLG **(15)** expressed by 282 accessions of the core collection planted in Saskatoon, SK and Morden, MB in 2009. Significant level of r was indicated by ** for $P < 0.01$.

The level of CLs expressed by individual accessions at the two locations were studied. The outliers were investigated to prove false positive results. Accession 'Hollandia' and 'Z 11637' were identified as lines that lack in CLs **1** and **3**. The two accessions were compared to their respective lines that were grown in Saskatoon and Morden every year from 2009 to 2010. Lack of CLs **1** and **3** in 'Hollandia' and 'Z 11637' were confirmed (Appendix E). The expression of gene AFSQ01016651.1 in these two accessions can be considered interesting. Levels of **9** in 'Hollandia' and 'Z 11637'were comparable to the mean of the group. On the other hand, CLs **1** and **3** could only be detected through HPLC/ESI-MS. These outcomes were consistent with the low expression level of total CL, as well as, ratio of CLs **1, 3, 9** to **6, 12, 15** by the two accessions.

4.4.4 Association of total cyclolinopeptide content with plant morphological traits

'CDC Bethune', 'Hanley', and 'Macbeth' were included in the core collection as comparisons against the 380 accessions. Comparison of three check accessions between the two locations showed the extraction consistency (Table 4.1). Generally, checks grown at Morden had higher levels of total CL than those grown in Saskatoon. 'Macbeth' expressed higher CLs when compared to the other two checks. The results were expected due to 'Macbeth' larger seed size and higher oil content (Duguid *et al*., 2003a). On the other hand, 'CDC Bethune' and 'Hanley' have medium seed size, medium oil content, and high yield when seeded in the brown and black soil zone of the prairie (Rowland *et al*., 2002; Duguid *et al*., 2003b). The expressions between gene #AFSQ01016651.1 to AFSQ01025165.1 were consistent across the accessions and growth locations.

Flax CLs content was compared in relation to both quantitative and qualitative morphological traits of the plant. Plant height, seed weight, total seed oil, and ALA concentration showed no correlation with total CL content (Appendix E). The Pearson correlation coefficients of CLs contents in accessions planted in Saskatoon and Morden were 0.0035 and 0.0007 for plant height, 0.0007 and 0.0035 for seed weight, 0.0245 and 0.0883 for total seed oil, and 0.02 and 0.0686 for ALA, respectively. Degree of stem branching, petal colour, and seed colour were not associated with total CL content in the accessions planted in either of the locations (Appendix F). These results agree with other studies that examined the association between mucilage (Diederichsen *et al*., 2006) and stem fibre content (Diederichsen and Ulrich, 2009) with other

seed characters. Long, non-branched stem, low seed yields, and small seeds are typical traits of fibre flax. However, Diederichsen and Raney (2008) pointed out that there was no association found between seed quantitative (plant height, total seed oil, and ALA) and qualitative (stem branching, petal colour, and seed colour) characters. No correlation between total CL content with qualitative and quantitative plant traits indicate that expression of CL in plant is independent and should not be affected by changes in these traits. Thus, flax breeders face no biological restriction when combining extreme qualitative and quantitative flax traits with desired CLs expression.

Seasonal condition on the plantation should be considered as an impact to the total CL content in either location. Lower mucilage content in flax has been linked to extreme humidity during the harvest period (Dorrell and Daun, 1980). Variation of rutin content in buckwheat due to solar radiation (Ohara *et al*., 1989), cropping season (Ohsawa and Tsutsumi, 1995), and soil types (Oomah and Mazza. 1996) have been reported by several studies. Similarly, Nahapetian and Bassiri (1976) observed strong influence of seasonal differences on phytate concentration in wheat. The environmental influence on the total CL content however, could not be investigated in this study. Oomah *et al*. (1996) proposed that environment might affect accessions differentially, such that metabolites expression is governed by the sensitivity of individual accession to environmental changes. Inappropriate technical handling of the accessions might as well led to seed contamination and hence, the variation of total CL content within individual accession.

4.5 Conclusion

The core collection of flaxseed grown in Saskatoon, SK and Morden, MB was screened to determine the profile and content of CL. The optimum extraction conditions for high throughput screening of CLs were established. Grinding prior to CL extraction was necessary to optimize recovery with aq. MeOH. Extraction efficiency increased at elevated temperature. The flaxseed extract was oxidized, in order to simplify the chromatographic analysis, and subsequently, quenched to stabilize Mso-containing CLs. Comparison of checks ‘CDC Bethune’, ‘Hanley’, and ‘Macbeth’ grown at two locations indicated the consistency of day-to-day extractions. The majority of accessions produced CL content within 1SD of the collection average. Accessions expressing highest and lowest peptide content were identified. Based on the predicted gene sequence, peptides encoded by NCBI gene AFSQ01016651.1 are expressed at higher

concentrations than peptides encoded by AFSQ01025165.1. No correlation was found between plant morphological traits (plant height, seed weight, stem branching, petal colour, seed colour, total seed oil, and ALA concentration) and plant CL content.

4.6 Connection to the next study

Within the flax core collection, 'Hollandia' and 'Z 11637' were identified as accessions expressing the lowest total CL content and the lowest ratio of peptide products from the two genes being studied. The accessions, grown over 3 growing seasons (2009-2011) in Saskatoon, SK and Morden, MB were re-analyzed using HPLC, ESI-MS and ESI-MS/MS and compared to the check 'CDC Bethune'.

CHAPTER 5

THREE NOVEL CYCLOLINOPEPTIDES FROM *Linum usitatissimum* L. 'HOLLANDIA' AND 'Z 11637'

5.1 Abstract

Three novel cyclolinopeptides (CLs) 1-Met-CLN, 1-Mso-CLN, and 1-Msn-CLN were isolated from seeds of *Linum usitatissimum* L. accessions 'Hollandia' (CN 98056, TMP 2181) and 'Z 11637' (CN 98150, TMP 2192). Extensive HPLC/ESI-MS and HPLC/ESI-MS/MS identified 1-Met-CLN [*m/z* 1072.6, *cyclo*-(Met-Leu/Ile-Leu/Ile-Pro-Pro-Phe-Phe-Leu/Ile-Leu/Ile)], 1-Mso-CLN [*m/z* 1088.6, *cyclo*-(Mso-Leu/Ile-Leu/Ile-Pro-Pro-Phe-Phe-Leu/Ile-Leu/Ile)], and 1-Msn-CLN [*m/z* 1104.6, *cyclo*-(Msn-Leu/Ile-Leu/Ile-Pro-Pro-Phe-Phe-Leu/Ile-Leu/Ile)]. Analysis of the methanolic extract of both accessions grown in Saskatoon, SK and Morden, MB in 3 growing seasons (2009-2011) confirmed their consistent presence.

5.2 Introduction

The parent peptides of known cyclolinopeptides (CLs) are encoded by 2 gene sequences. The NCBI gene AFSQ01016651.1 codes for 3 parent peptides (CLA, 1-Met-CLB and 1-Met-CLE), while the sequence AFSQ01025165.1 codes for the other 3 parent peptides (1-Met-CLD, 1-Met,3-Met-CLF and 1-Met,3-Met-CLG) (Sayers *et al*., 2009). A search of the published sequences of flax 'CDC Bethune' using the coding sequence of both AFSQ01016651.1 and AFSQ01025165.1 identified a number of additional related sequences. Close examination of these sequences indicated that they may produce peptides. If these sequences are expressed in flaxseed it is possible that there are additional peptides present in flaxseed products. It is proposed that extracts of flaxseed meal be produced and screened to determine if any additional cyclic peptides can be identified in flax using mass spectrometry.

Mass spectrometry (MS) is an analytical tool that utilizes the mass to charge ratio (*m/z*) of ionized atoms to determine the molecular mass of a compound. When the mass is determined by

a high precision spectrometer (TOF-MS), it is often possible to determine the empirical formula of a compound. Other MS devices allow the fragmentation of a molecule and passage of the molecular fragments through the spectrometer. This latter method requires two stages of mass spectrometry and is called MS/MS. MS and MS/MS have been used widely in various fields, including pharmaceutical, clinical, biotechnology, environmental, geological, etc. In biochemistry, this tool has been used for molecular weight measurement, amino acid sequencing, oligonucleotide sequencing, protein structure analysis, and reaction monitoring. Mass spectrometry offers accuracy of 0.01% and 5ppm of the total molecular mass of the sample for biomolecules and small organic molecules respectively, which makes this tool sensitive enough to detect minor mass changes. Three fundamental parts of mass spectrometry are ionization source, analyzer, and detector. Once inside the ionization compartment, the vaporized sample is reacted with a stream of electrons produced by an electrically heated metal coil. Ionized samples are then separated according to the electron charge (z) and their overall mass (m). The ion current will then be monitored, amplified, and stored in the form of a mass spectrum.

The use of MS for detecting CLs has been recorded (Stefanowicz, 2001; Brühl *et al.*, 2007). The detection method of CLs using HPLC-MS and HPLC-MS/MS was described by Stefanowicz (2001). Following extraction and base hydrolysis, extract of ground flax was evaporated and further eluted with ethyl acetate. The sample again was evaporated, re-constituted in 10 mM solution of ammonium acetate in methanol, and injected to a Finnigan MAT TSQ-700 MS, equipped with ESI source at flow rate of 2.0 $\mu L \cdot min^{-1}$. The study confirmed the presence of known peptides CLA, 1-Met-CLB, 1-Mso-CLD, and 1-Mso-CLE, reported previously by Morita *et al.* (1999). Precursors of the two latter peptides, 1-Met-CLD, and 1-Met-CLE were detected and sequenced. In addition, Stefanowicz (2001) also reported the presence of two novel CLs, identified as 1-Met,3-Met-CLF and 1-Met,3-Met-CLG. The sequences, *cyclo*-(Met-Leu-Mso-Pro-Phe-Phe-Trp-Val) and *cyclo*-(Met-Leu-Met-Pro-Phe-Phe-Trp-Ile), respectively were proposed on the basis of collision induced disassociation tests and homology to 1-Met-CLD. A more recent study by Brühl *et al.* (2007) utilized MS as a means to confirm the molecular formula of purified 1-Mso-CLE, the peptide contributing bitter flavour in oxidized flax oil. Following isolation of fraction containing the bitter compound, a fraction of the concentrated bitter compounds was dissolved in methanol and injected to a Bruker Mikro TOF using ESI with methanol/water (1/1, v/v) at a flow rate of 200 $\mu L \cdot min^{-1}$. High sensitivity and small sample

preparation were the main reason to choose MS for early detection and identification mean of novel peptides. On the other hand, research involving MS suffered difficulties in distinguishing amino acid residues with similar molecular weights (Ile and Leu), as well as, determining configuration of amino acids. Other analytical methods suitable for further confirmation of CLs include NMR, infrared spectroscopy, circular dichroism spectroscopy, high resolution fast atom bombardment mass spectrometry (HR-FABMS) and FTIR (Brewster and Bovey, 1971; Naider *et al*, 1971; Morita *et al*., 1999; Matsumoto *et al*., 2001; Brühl *et al*., 2007).

The lack of CLA and 1-Mso-CLB in *L. usitatissimum* 'Hollandia' and 'Z 11637' was reported in the previous study. This study originally was designated to confirm the earlier findings through HPLC/ESI-MS and HPLC/ESI-MS/MS. However, unidentified peak at the chromatograms of Hollandia and Z 11637 extracts suggested the presence of novel CLs. 'Hollandia' and 'Z 11637', regenerated in 2 locations and 3 growing seasons were analyzed. HPLC/ESI-MS and HPLC/ESI-MS/MS were utilized to determine the molecular weight, and sequences of novel CLs.

5.3 Experimental

5.3.1 Reagents and samples

HPLC-grade acetonitrile (CH_3CN) and methanol (MeOH) were purchased from Fischer Scientific (Fair Lawn, NJ, USA). Water (H_2O) was purified to 18.2 MΩ cm on a Milli-Q Integral system (Millipore, Molsheim, France). All chemicals used were weighed using analytical balance (MSA225S000DU, Sartorius, Germany). Seed materials used were 'Hollandia' (CN 98056, TMP 2181), 'Z 11637' (CN 98150, TMP 2192), and 'CDC Bethune' from the flax core collection, grown at Kernen Farm, Saskatoon, SK and Morden Research Centre, Morden, MB planted in 2009, 2010, and 2011.

5.3.2 Sample Preparation

Extraction of CLs from flaxseed was conducted according to chapter 4.

5.3.3 HPLC apparatus

Chromatographic separations of flaxseed extract through HPLC, HPLC/ESI-MS, and HPLC/ESI-MS/MS were achieved according to section 3.3.2.

5.4 Results and Discussion

L. usitatissimum L. 'Hollandia' and 'Z 11637' were compared against 'CDC Bethune', the check accession in the flax core collection. HPLC chromatogram of the oxidized 'CDC Bethune' extract showed elution of CLs **12**, **15**, **3**, **9**, **6**, and **1** at retention time of 2.5, 2.7, 2.9, 3.2, 3.5, and 4.3 (Figure 5.1A). An additional peak at a retention time of 3.1 min was observed in the chromatograms of CL extracts from 'Hollandia' and 'Z 11637'. Absence of CLs **1** and **3** was noted on these accessions. Accessions 'Hollandia', 'Z 11637' and 'CDC Bethune' from two locations, Saskatoon and Morden, were re-extracted for CL analyses to confirm this observation. The extracts were analyzed with and without the addition of hydrogen peroxide (H_2O_2) to determine if the new peak is an oxidation product or a new peptide unaffected by the oxidation process. HPLC analyses of the unoxidized extract of 'CDC Bethune' showed the occurrence of **2**, **14**, and **5** at retention time 3.9, 4.3, and 4.7 (Figure 5.1B). CLs **1** and **8** eluted simultaneously at 4.3. Besides the known peptides, chromatograms of the unoxidized extract of 'Hollandia' and 'Z 11637' displayed an additional peak at retention time of 4.2 min. The absence of **3** was supported by the absence of **2** in the unoxidized extract of 'Hollandia' and 'Z 11637'. The absence of **1** could not be confirmed due to co-elution of **1** with **8** in the HPLC method used. HPLC analysis of 'Hollandia' and 'Z 11637' grown at two locations in three years confirmed the presence of the new peaks in consistent absorbance levels (data not shown).

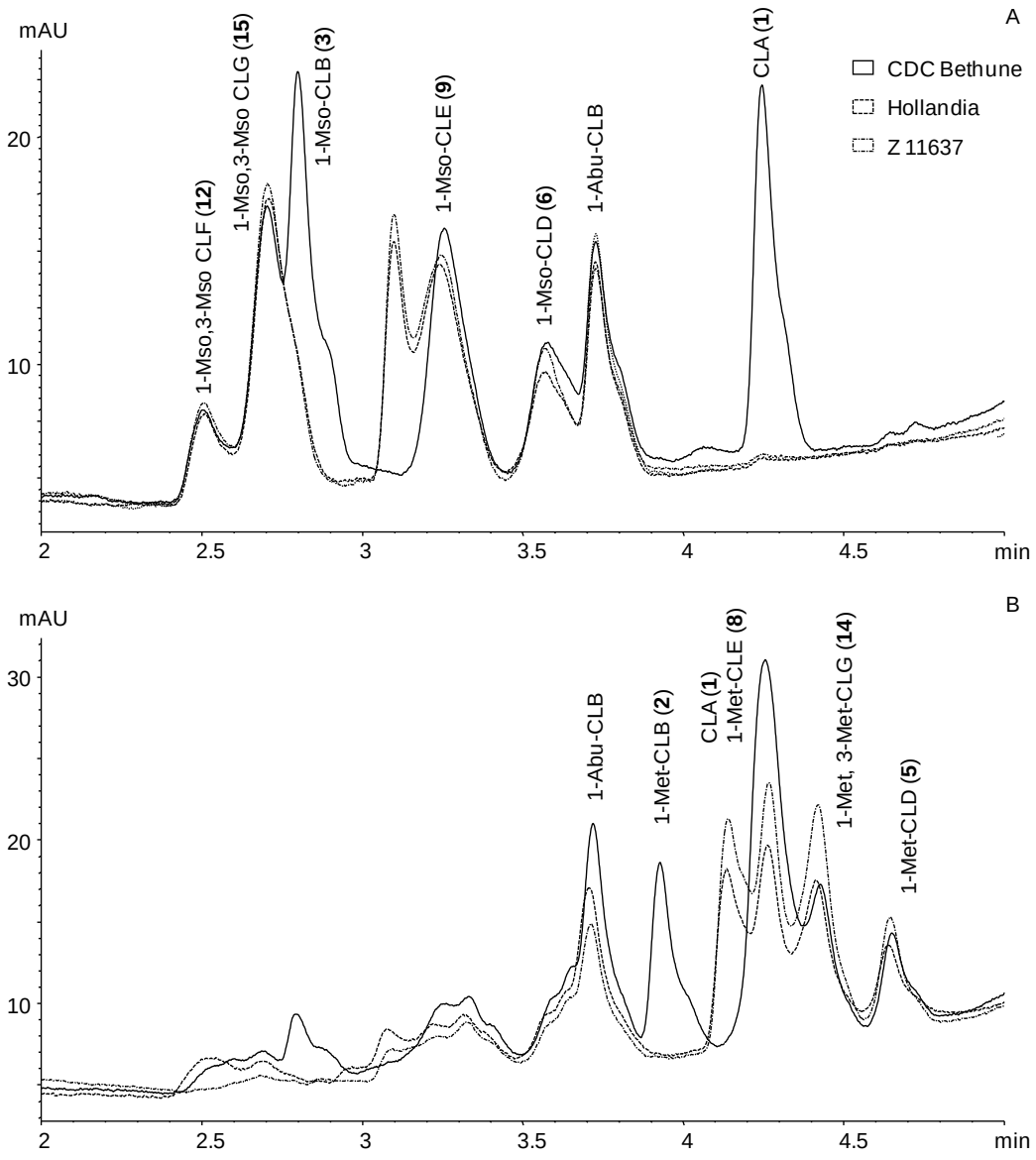

Figure 5.1 Chromatograms of (A) oxidized CL extracts and (B) unoxidized CL extracts from *L. usitatissimum* 'CDC Bethune', 'Hollandia' and 'Z 11637'.

The oxidized and unoxidized extracts of 'Hollandia', 'Z 11637' and 'CDC Bethune' were subjected to an extensive HPLC/ESI-MS and HPLC/ESI-MS/MS. The analysis confirmed the absence of CLs **1** and **3** in the extracts of 'Hollandia' and 'Z 11637'. The unidentified peak occurring in unoxidized extract possessed *m/z* at 1072.6 (Figure 5.2). Two peaks appeared in the HPLC/ESI-MS chromatograms of oxidized extract of Hollandia' and 'Z 11637' were identified as *m/z* 1088.6 and *m/z* 1104.6. The ion at *m/z* 1088.6 was found in the unoxidized extracts, although in low level. The putative fragmentation pattern of the ion at *m/z* 1072.6 as *cyclo*-(Met-Leu/Ile-Leu/Ile-Pro-Pro-Phe-Phe-Leu/Ile-Leu/Ile) (Figure 5.3), while ion at *m/z* 1088.6 and 1104.6 were identified as *cyclo*-(Mso-Leu/Ile-Leu/Ile-Pro-Pro-Phe-Phe-Leu/Ile-Leu/Ile) and *cyclo*-(Msn-Leu/Ile-Leu/Ile-Pro-Pro-Phe-Phe-Leu/Ile-Leu/Ile), respectively. The additional 16 Da in molecular weight of ion at *m/z* 1088.6 corresponded to the conversion of Met in ion at *m/z* 1072.6 to Mso in ion at *m/z* 1088.6. Similar observation was noted on further additional 16 Da in ion at *m/z* 1104.6, in which Msn was detected. Hence, the putative amino acid sequences and molecular weight of these three ions indicated that oxidation of CL at *m/z* 1072.6 produced CLs at *m/z* 1088.6, where Met was converted to Mso, and 1104.6, where Mso was further converted to Msn.

The general scheme of peptide fragmentation agrees with that reported previously (Morita *et al*., 1999; Matsumoto *et al*., 2001; Stefanowicz, 2001) in which the opening of cyclic peptide ring take place at the amide nitrogen of Pro residue. Following the main ring opening, losses of amino acids occur at the C-terminal of the linear peptide.

The gene sequence of these peptides is still unknown, as the available gene sequence published in www.linum.ca is based only on 'CDC Bethune'. Continuing the proposed naming pattern of CLs, the peptides with *m/z* 1072.6, *m/z* 1088.6 and *m/z* 1104.6 were designated as 1-Met-CLN, 1-Mso-CLN and 1-Msn-CLN. The amino acids sequences of 1-Met-CLN, 1-Mso-CLN and 1-Msn-CLN were established according to the homology of Leu and Ile position with known peptides CLs **1**, **2**, **3**, and **4**. The sequence Pro-Pro-Phe-Phe observed in these four peptides was also present in 1-Met-CLN, 1-Mso-CLN and 1-Msn-CLN. Homology of 1-Met-CLN, 1-Mso-CLN and 1-Msn-CLN to CLs **1** and **2** is worth noting since the known immunosuppressive ability of CLs **1, 2** and **4** (Wieczorek, *et al.*, 1991; Morita *et al.*, 1999; Morita and Takeya, 2010) might give an indication on possible biological activity of the novel peptides.

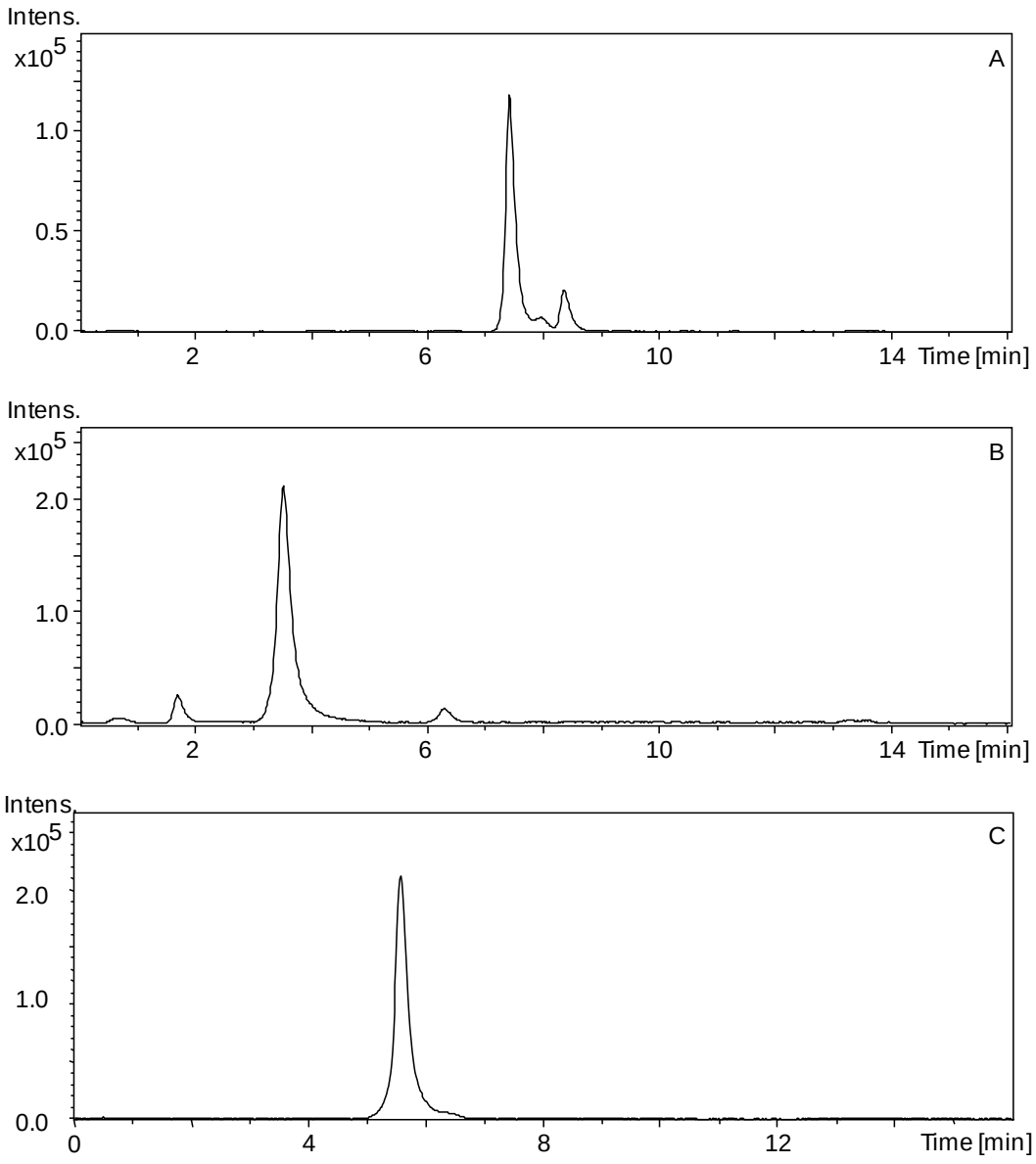

Figure 5.2 Extracted ion chromatograms of (A) *m/z* 1072.6, (B) *m/z* 1088.6, and (C) *m/z* 1104.6 in the methanol extract of *L. usitatissimum* L. 'Hollandia'

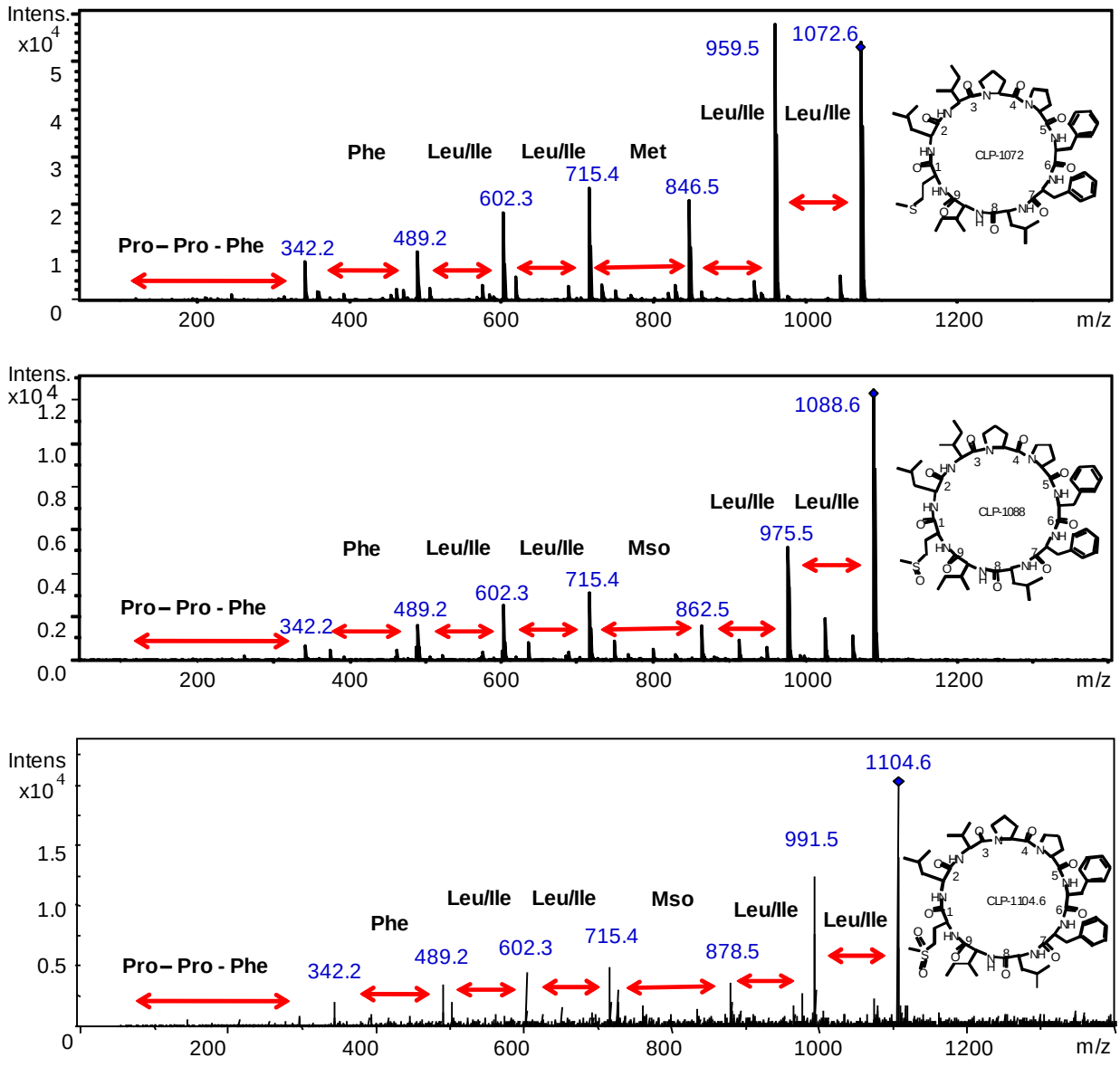

Figure 5.3 HPLC/ESI-MS/MS spectra and the proposed amino acid sequence for 1-Met-CLN (*m/z* 1072.6), 1-Mso-CLN (*m/z* 1088.6), and 1-Msn-CLN (*m/z* 1104.6)

5.5 Conclusion

Three novel 1-Met-CLN [*m/z* 1072.6, *cyclo*-(Met-Leu/Ile-Leu/Ile-Pro-Pro-Phe-Phe-Leu/Ile-Leu/Ile)], 1-Mso-CLN [*m/z* 1088.6, *cyclo*-(Mso-Leu/Ile-Leu/Ile-Pro-Pro-Phe-Phe-Leu/Ile-Leu/Ile)], and 1-Msn-CLN [*m/z* 1104.6, *cyclo*-(Msn-Leu/Ile-Leu/Ile-Pro-Pro-Phe-Phe-Leu/Ile-Leu/Ile)] were identified in accessions 'Hollandia' and 'Z 11637' of the flax core collection. Addition of H_2O_2 during the extraction process oxidized Met to Mso and to Msn. A complete structure elucidation of 1-Met-CLN, 1-Mso-CLN, and 1-Msn-CLN may be conducted when sufficient material is obtained.

CHAPTER 6
GENERAL DISCUSSION

Cyclolinopeptides (CLs), a group of naturally occurring cyclic peptides present in flaxseed, have shown potential immunosuppressive, cytoprotective, and antimalarial activities. The Flax World Core Collection, which represents the widest variation of flax gene resources, was screened to determine the concentration and composition of CLs. A high throughput extraction method, conducted in a 96-well plate format was developed. Extraction involved incubation of ground (degummed) flaxseed at 60 ºC for 2 h in 80% aq. MeOH (1:8, w/v). After separation of the solids from the supernatant the filtered extract was oxidized and quenched. Quenched flaxseed extract was passed through a monolithic C_{18}-bonded silica gel chromatographic column (CSR), with a mobile phase that consisted of H_2O–CH_3CN (70:30 30:70 in 4 min, to 10:90 in 0.5 min, and to 70:30 in 0.5 min) at a flow rate of 2.0 $mL \cdot min^{-1}$ with a total run time of 6.0 min. The longevity of the monolithic CSR column observed over thousands of injections demonstrated that this column was suitable for high-throughput CL screening. High throughput extraction and rapid HPLC analysis of CL enabled analysis of 2000 samples (380 accessions and 120 checks) in 22 days.

Profiles of CLs vary among the accessions analyzed. The majority of flax core accessions expressed CLs in amount within 1SD from the collection average, with 'AC Watson' flax identified as the accession expressing the highest level of total CL at >2SD above the population mean. Conversely, 'Hollandia' and 'Z 11637' flax, both originating from collections of the Netherlands expressed the lowest content of CL. The lack of parent peptides CLA and 1-Met-CLB in these accessions is accompanied by the presence of novel peptides 1-Met-CLN, 1-Mso-CLN, and 1-Msn-CLN. No correlations were observed between either CL content and plant morphological traits (height, stem branching, petal colour, and seed colour) or chemical traits (total seed oil and ALA content) were evident. Independence of CL production from other flax traits indicates that expression of CL would not be affected by any change in other traits.

Therefore, a breeding program that would combine improved flax morphology and oil content with high or low CL content would likely face no biological restriction.

CHAPTER 7
GENERAL CONCLUSION

High throughput screening is widely used in plant breeding programs for selection of lines with improved traits. In this thesis, a method for high throughput screening of CL in flax accessions from the world core collection was developed. Extraction of flaxseed in 96-well plate format followed by rapid HPLC analysis with monolithic columns enabled fast and accurate analysis of flaxseed CL concentration and composition. Other plants metabolites, including but not limited to mucilage, polyphenols, lignans and cyanogenic glycosides can also be studied employing similar protocols.

Ultra performance liquid chromatography (UPLC) has recently emerged as an improved rapid high resolution HPLC method. In comparison to the conventional HPLC, UPLC typically offers significant reduction of analysis time often with improved separating power. The studies in this thesis show that the combination of monolithic columns and conventional HPLC enabled similar reduction in analysis time without sacrificing resolution. Separation of 7 CLs was achieved in a total analysis time of 1.5 min. The monolithic column was proven to be robust, as repeated injections of thousands of samples did not affect either backpressure or resolution. Therefore, a conventional HPLC may be used for high throughput screening of a large population of plant accessions when a rapid protocol is developed on a suitable monolithic column.

The study also included the repeated analysis of hundreds of check accessions that enabled the study of the experiment variability. Coefficient of variation (CV) is a measure of this variability. Small CV values of individual peptide concentration of check accessions ‘CDC Bethune’, ‘Hanley’, and ‘Macbeth’ illustrate stability and uniformity of the extraction and analysis method. On the other hand, the high throughput method developed in this thesis is sensitive enough to detect both the loss of a peptide and the occurrence of unique peptides within the samples. Among 380 accessions of flax world core collection, 2 accessions are identified as containing unique peptides. ‘Hollandia’ and ‘Z 11637’ flax do not contain the previously reported parent peptides, but express novel peptides.

Plant peptide content and composition are both elements of plant phenotype. As with other elements of phenotype, peptide content and composition are, therefore, determined by genotype and environmental conditions. Knowledge of the interaction of genotype with environment may assist flax breeding programs and ultimately, enhance production, yield, and value of flax. In the future, further data collection from the core collection planted in 2010 and 2011 will be compared with those generated from this study (2009) in order to establish the environmental effect on CL content and composition.

The discovery of novel peptides has introduced a potential new area of flax peptide research. This unstudied compound may be isolated in multigram quantities to enable structure elucidation and assays of biological activity. The mechanism by which these novel peptides arose and peptides CLA and 1-Met-CLB were lost may be revealed when a whole genome shotgun sequences of accession 'Hollandia' and 'Z 11637' are available.

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APPENDIX A
LIST OF CHEMICALS

Purchased from EMD Chemicals, Inc. (Gibbstown, NJ)

Acetic acid, Glacial	GR ACS grade
Potassium iodate	GR ACS grade
Sodium hydroxide	GR ACS grade

Purchased from Fischer Scientific International, Inc. (Fair Lawn, NJ)

Acetonitrile	HPLC grade
Methanol	HPLC grade

Purchased from Sigma-Aldrich (St. Louis, MO)

Formic acid	HPLC grade
Hydrogen peroxide	GR ACS grade
Sodium periodate	GR ACS grade
Sodium thiosulfate	99%, ReagantPlus® grade

APPENDIX B

QUALITY REPORTS OF CYCLOLINOPEPTIDE STANDARDS

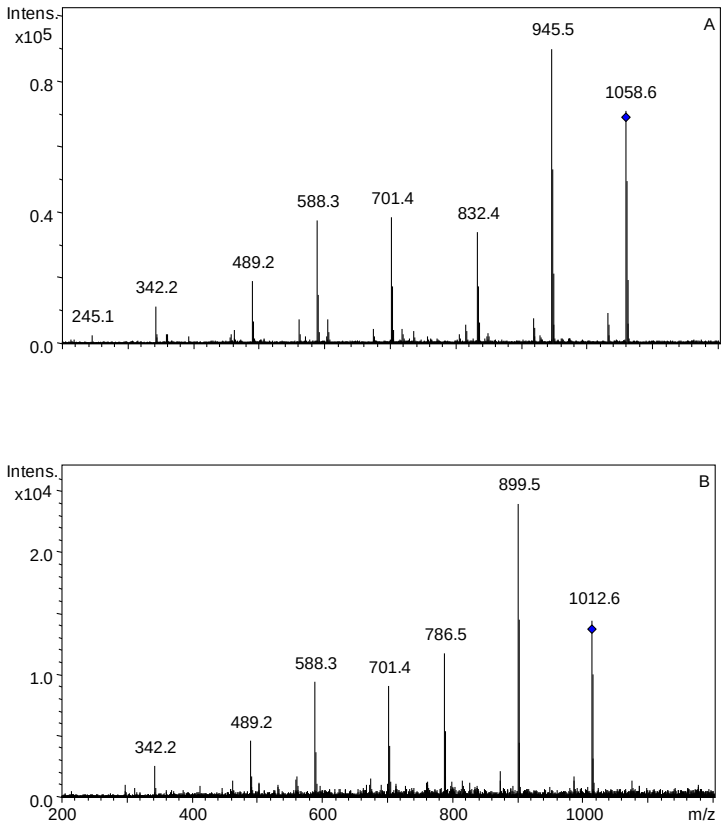


Figure B.2. HPLC-MS/MS spectra of standards (A) 1-Met-CLB (**2**), (B) 1-Abu-CLB, (C) 1-Mso-CLB (**3**), and (D) 1-Msn-CLB (**4**) (cont'd)

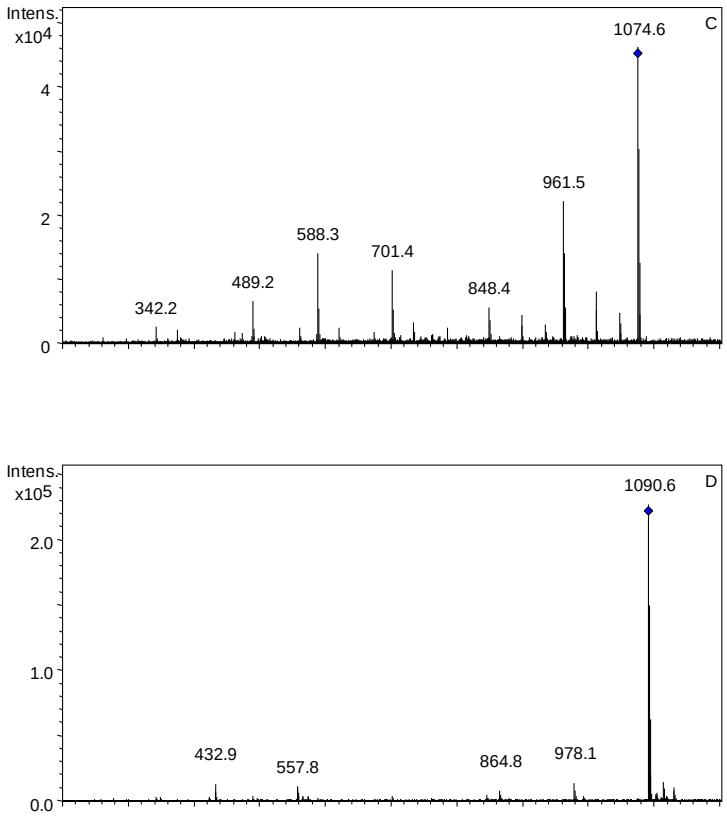


Figure B.2. HPLC-MS/MS spectra of standards (A) 1-Met-CLB (**2**), (B) 1-Abu-CLB, (C) 1-Mso-CLB (**3**), and (D) 1-Msn-CLB (**4**) (cont'd)

APPENDIX C

CHROMATOGRAPHIC SEPARATION OF CYCLOLINOPEPTIDES

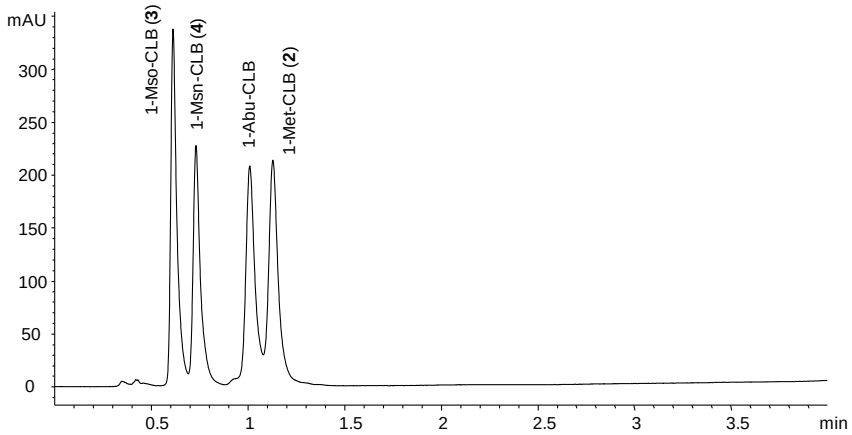


Figure C.1 Chromatogram of a mixture of CLs and 1-Abu-CLB obtained using Chromolith® SpeedROD. Gradient elution is as listed under Performance/High Resolution in Table 3.2 at flow rate of 2.0 mL·min^{-1}.

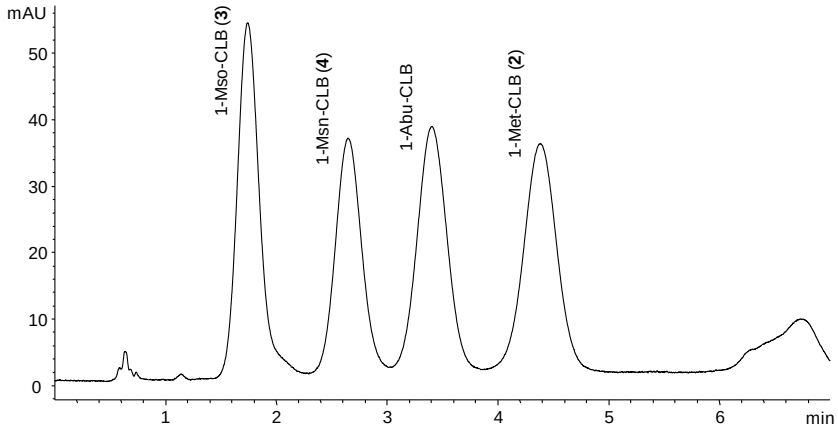


Figure C.2 Chromatogram of a mixture of CLs and 1-Abu-CLB obtained using POROS at a flow rate of 2.0 mL·min^{-1} with the following gradient elution: 0 min 35% B, 5.0 min 45% B, 5.5 min 90% B, 6.0 min 35% B, 7.0 min 35% B.

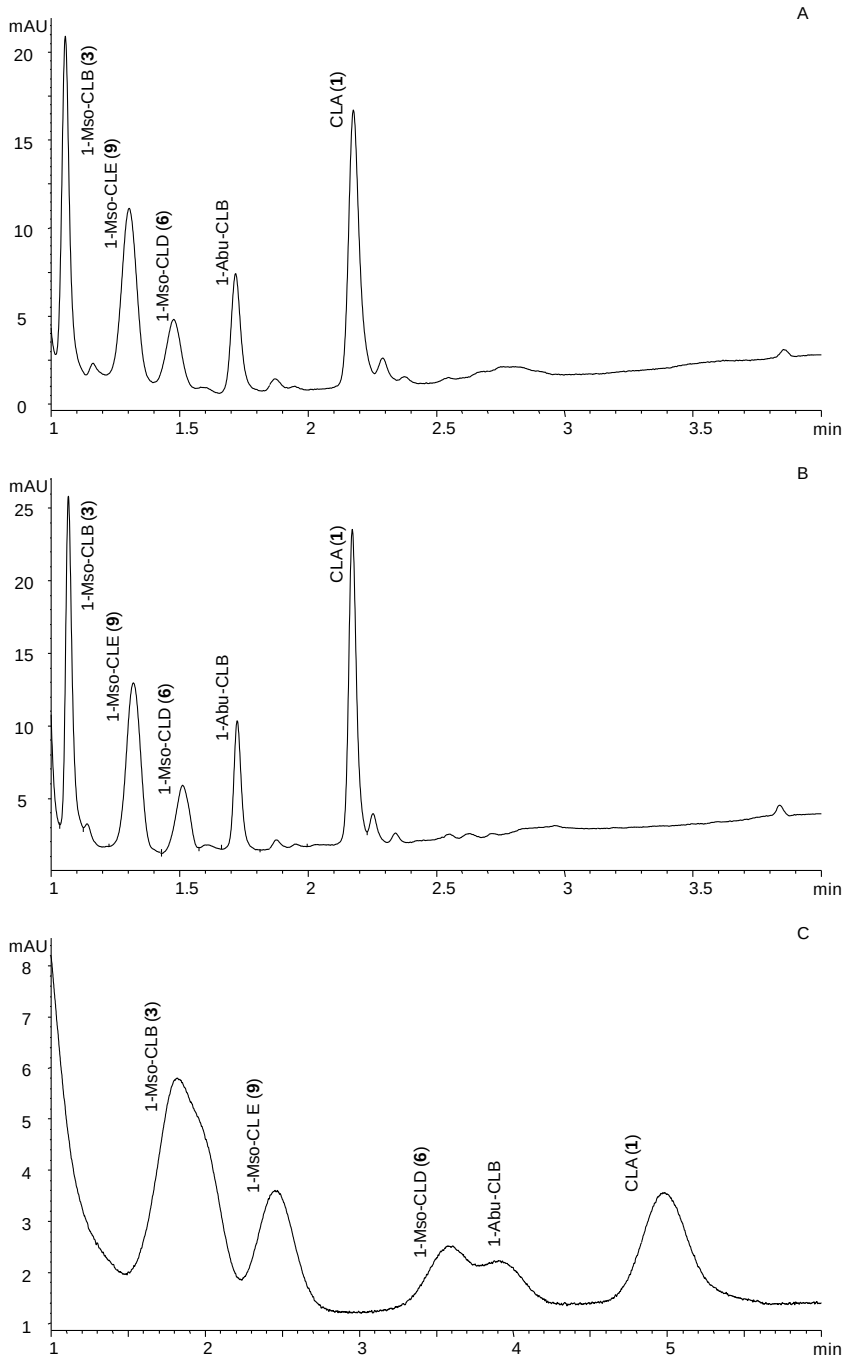


Figure C.3 Chromatograms of methanol extract of flax seed showing mixture of 1-Mso,3-Mso-CLF (**12**), 1-Mso,3-Mso-CLG (**15**), 1-Mso-CLB (**3**), 1-Mso-CLE (**9**), 1-Mso-CLD (**6**), and CLA (**1**) with internal standard, 1-Abu-CLB obtained using different columns: (A) Chromolith® Performance, (B) Chromolith® High Resolution, and (C) POROS. Gradient elution is as listed under SpeedROD in Table 3.2.

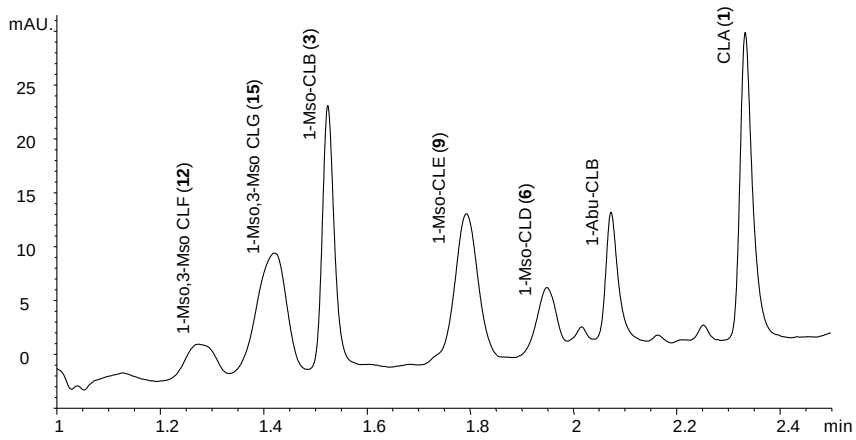


Figure C.4 Chromatogram of methanol extract of flax seed showing mixture of 1-Mso,3-Mso-CLF (**12**), 1-Mso,3-Mso-CLG (**15**), 1-Mso-CLB (**3**), 1-Mso-CLE (**9**), 1-Mso-CLD (**6**), and CLA (**1**) with internal standard, 1-Abu-CLB obtained using Chromolith® High Resolution at a flow rate of 2.0 mL·min^{-1} with the following gradient elution: 0 min 50% B, 1.5 min 90% B, 1.6 min 90% B, 1.7 min 50% B, 2.5 min 50% B.

APPENDIX D

PROFILE OF OBSERVED CYCLOLINOPEPTIDES IN CORE COLLECTION

Table D.1 Profile of CLs (mAU) in oxidized methanol extract of flax accessions of core collection grown in Saskatoon, SK in 2009

CN	TMP	1-Mso,3-Mso-CLF	1-Mso,3-Mso-CLG	1-Mso-CLB	1-Mso-CLE	1-Mso-CLD	CLA	Total	Ratio
101373	TMP-13173	9.2	26.2	37.3	37.8	11.5	40.3	162.2	2.5
98683	TMP-8209	8.7	24.7	30.5	33.1	10.7	34.2	141.9	2.2
	CDC Bethune	12.0	37.8	34.4	35.4	15.3	36.0	171.0	1.6
	TMP 2681-1				no seed material				
100884	TMP-1459	7.1	21.7	30.0	33.2	9.4	33.3	134.7	2.5
101382	TMP-13182	8.7	26.8	30.8	34.3	10.7	32.9	144.2	2.1
98741	TMP 8273-2	10.4	30.4	40.3	41.7	11.8	41.4	176.0	2.3
	CDC Bethune	12.0	37.8	30.8	31.0	14.3	31.0	156.9	1.4
30861	PGR-1726	7.5	23.5	36.3	42.3	10.8	39.4	159.8	2.8
97402	TMP 2983-13	13.1	41.0	40.6	47.0	19.2	43.3	204.3	1.8
97404	TMP-2985	8.2	24.8	28.4	34.1	10.7	31.9	138.1	2.2
98710	TMP 8238-10	11.2	35.3	37.9	43.6	13.1	40.6	181.7	2.0
	CDC Bethune	11.7	29.6	40.5	43.5	12.7	43.2	181.2	2.4
	TMP-9996				no seed material				
98566	TMP 2945-7	5.9	19.5	37.9	38.0	7.3	38.2	146.8	3.5
96846	TMP-8374	6.1	17.8	30.8	31.5	6.8	32.4	125.4	3.1
98012	TMP-2443	5.1	17.0	27.9	39.2	27.1	31.0	147.2	2.0
	CDC Bethune	11.9	36.4	35.4	60.0	15.8	38.2	197.6	2.1
97180	TMP-2289	5.9	21.0	27.4	33.3	11.4	32.9	132.0	2.4
97483	TMP 7553-6	8.2	26.0	31.6	34.1	9.8	34.0	143.6	2.3
18993	TMP-1162	11.2	34.9	43.3	58.3	13.3	46.1	207.1	2.5
101535	TMP-9980	9.8	30.6	45.8	47.2	12.6	47.2	193.3	2.6
	CDC Bethune	12.7	32.7	39.6	42.8	14.4	41.1	183.2	2.1
97768	TMP-2343	7.5	24.1	40.7	40.8	10.0	40.5	163.6	2.9
97921	TMP-7912	7.0	23.0	28.2	32.0	9.0	30.7	129.9	2.3
98231	TMP-8533	6.5	20.9	29.9	32.4	9.2	31.5	130.5	2.6
98440	TMP-2887				no seed material				
	CDC Bethune	14.4	41.8	41.2	43.8	16.5	42.6	200.2	1.8
100952	TMP-1654	12.9	39.1	43.4	46.6	15.1	45.7	202.8	2.0
97404	TMP 2985-5	10.9	35.4	30.8	35.3	13.2	32.5	158.2	1.7
101407	TMP-13214	10.1	32.1	30.4	33.7	11.5	33.0	150.8	1.8
98712	TMP-8240	8.6	26.0	35.3	39.0	11.0	36.0	155.9	2.4
	CDC Bethune	17.6	56.9	56.3	56.6	22.5	55.5	265.4	1.7
97397	TMP-2979	7.5	23.7	30.8	39.2	9.1	33.8	144.1	2.6

101560	TMP-13679	6.9	21.3	34.6	39.3	8.3	35.1	145.4	3.0
100790	TMP-11337	7.6	22.3	47.4	46.8	9.8	50.6	184.4	3.6
101094	TMP-1833	9.9	31.8	34.2	33.1	11.8	35.0	155.7	1.9
	CDC Bethune	10.6	32.4	40.5	42.2	11.9	43.6	181.2	2.3
98039	TMP-2469	7.3	24.1	27.3	32.9	10.3	28.4	130.2	2.1
101052	TMP-1791	8.9	27.5	35.1	38.9	12.4	35.4	158.1	2.2
100805	TMP-1365	7.6	24.8	31.2	39.1	10.0	34.9	147.7	2.5
98767	TMP-8301	8.2	26.6	31.8	33.5	10.7	32.3	143.1	2.1
	CDC Bethune	10.5	33.3	37.7	42.4	13.4	41.4	178.7	2.1
101329	TMP-13128	8.4	27.1	29.5	33.6	10.4	31.1	140.1	2.1
97531	TMP-2161				no seed material				
98566	TMP-2945				no seed material				
	TMP 2945-4				no seed material				
	CDC Bethune	11.1	34.5	39.3	50.0	13.5	43.7	192.0	2.3
98926	TMP 2224-10				no seed material				
96911	TMP-8409	8.5	27.5	37.6	40.2	11.5	39.9	165.2	2.5
97351	TMP-8236	8.4	27.3	30.0	30.8	10.6	31.2	138.3	2.0
	TMP-1777				no seed material				
	CDC Bethune	15.1	48.5	43.2	42.2	20.0	43.6	212.6	1.5
98829	TMP-2156	9.8	32.5	36.3	38.7	12.1	37.6	167.0	2.1
96991	TMP-2087	6.5	21.1	32.0	36.5	8.2	32.8	137.1	2.8
98014	TMP-2445	8.5	26.4	31.0	35.0	10.4	33.6	145.0	2.2
19159	TMP-1354	8.6	27.4	41.6	52.6	11.4	46.1	187.6	3.0
	CDC Bethune	11.0	32.6	38.9	41.0	13.9	43.5	180.9	2.1
	Hanley	12.0	35.1	33.2	41.0	12.7	36.8	170.7	1.9
	Macbeth	12.8	39.0	39.6	49.9	14.9	44.6	200.8	2.0
97463	TMP-7537	10.6	34.2	35.0	31.8	13.4	33.3	158.3	1.7
	Macbeth	13.0	37.4	34.9	42.6	14.6	39.2	181.7	1.8
	CDC Bethune	12.0	37.8	36.5	36.7	15.7	37.8	176.5	1.7
	Hanley	8.1	23.9	60.6	56.5	9.3	61.2	219.6	4.3
97665	TMP 7702-5	11.8	37.9	40.5	43.6	13.7	40.4	187.9	2.0
97103	TMP-8560	9.1	28.9	30.4	33.5	11.4	32.1	145.4	1.9
101307	TMP-10896				no seed material				
	CDC Bethune	10.9	33.0	34.6	35.6	13.8	36.7	164.7	1.9
	TMP-8376				no seed material				
101510	TMP-9952	5.8	18.3	39.4	40.9	7.5	42.8	154.6	3.9
97886	TMP-2361	13.3	42.4	42.9	42.0	16.0	40.2	196.9	1.7
19007	TMP-1440	5.7	19.2	27.8	29.7	10.7	27.0	120.2	2.4
	CDC Bethune	9.7	30.5	36.7	38.7	13.4	41.1	170.1	2.2
33399	PGR-5050	9.4	29.0	27.5	29.8	11.4	29.6	136.7	1.7
101298	TMP-10886	8.9	27.3	43.2	39.7	12.2	43.2	174.4	2.6
98773	TMP-8307	10.2	31.0	39.2	41.1	12.4	39.9	173.8	2.2
101115	TMP-1854	10.9	33.4	32.7	30.9	12.6	32.5	153.0	1.7

	CDC Bethune	10.6	32.9	39.5	40.9	12.7	43.9	180.4	2.2
101289	TMP-10877				no seed material				
100795	TMP-10250				no seed material				
101406	TMP-13213	5.6	16.9	19.7	19.3	6.8	20.7	88.9	2.0
98007	TMP-2438	8.2	26.2	23.6	24.5	10.6	24.0	117.2	1.6
	CDC Bethune	11.2	34.0	36.2	39.2	14.5	39.7	174.8	1.9
101469	TMP-10112				no seed material				
97475	TMP-7544	10.9	32.8	30.5	40.2	13.8	15.0	143.2	1.5
97613	TMP-7666	12.0	39.7	43.4	40.0	16.2	39.5	190.9	1.8
18983	TMP-1152	11.8	36.2	38.9	38.9	14.2	38.6	178.8	1.9
	CDC Bethune	13.3	44.0	39.9	39.3	17.1	39.8	193.4	1.6
101395	TMP-13195				no seed material				
101454	TMP-10109	10.3	31.1	31.9	45.4	11.5	37.6	167.7	2.2
97980	TMP-2412	6.3	19.6	25.3	26.3	8.3	27.7	113.5	2.3
101417	TMP-1920	8.6	27.7	28.3	31.7	11.3	29.8	137.4	1.9
	CDC Bethune	12.7	36.0	40.8	43.3	14.2	43.0	190.0	2.0
98398	TMP-2845	6.3	18.0	19.4	20.4	7.3	21.1	92.5	1.9
97639	TMP-7684	8.9	27.5	32.2	37.2	10.5	35.5	151.8	2.2
	Macbeth	18.6	44.3	38.2	46.4	17.4	41.9	206.8	1.6
	Hanley	9.8	33.9	31.1	31.5	14.5	30.2	151.1	1.6
	CDC Bethune	11.6	33.9	33.5	35.5	14.6	34.3	163.5	1.7
18991	TMP-1160	13.9	40.9	47.7	47.0	16.8	47.9	214.3	2.0
97147	TMP-8570	9.9	28.4	36.8	41.0	17.6	39.5	173.3	2.1
97306	TMP-2938				no seed material				
101580	TMP-10121	8.4	25.1	50.9	54.7	10.0	55.3	204.3	3.7
	CDC Bethune	9.9	29.7	36.3	38.0	11.9	39.0	164.8	2.2
101451	TMP-10033	8.9	25.8	32.7	35.6	10.2	34.7	147.8	2.3
97452	TMP-7529	8.2	26.1	16.9	17.9	10.6	17.2	96.9	1.2
100770	TMP-11317	13.6	39.6	38.1	38.6	16.0	39.6	185.6	1.7
101053	TMP-1792	12.9	37.6	31.9	33.1	15.4	34.1	165.0	1.5
	CDC Bethune	10.8	33.7	35.2	40.3	12.9	38.6	171.5	2.0
97334	TMP-8148	9.9	29.7	39.5	39.5	10.6	40.9	170.2	2.4
98275	TMP-2535	13.1	39.8	42.5	39.8	15.7	42.1	193.1	1.8
100799	TMP-8366	7.4	23.7	44.2	45.7	28.9	46.6	196.4	2.3
101565	TMP-10004	10.9	28.9	29.0	37.7	12.9	34.0	153.5	1.9
	CDC Bethune	12.2	38.4	41.5	48.5	15.2	46.0	201.9	2.1
97689	TMP-7723	10.4	34.0	38.0	38.4	15.6	33.3	169.7	1.8
18980	TMP-1070	10.2	26.8	32.2	36.7	12.5	34.9	153.4	2.1
97307	TMP-2939				no seed material				
	CDC Mons	11.2	32.6	38.5	42.1	13.5	41.2	179.2	2.1
	CDC Bethune	12.1	34.7	36.7	38.8	14.0	38.9	175.2	1.9
98057	TMP-2655	7.1	25.9	33.6	44.1	12.8	41.7	165.2	2.6
101594	TMP-10125	9.7	29.5	41.6	46.4	12.6	40.0	179.8	2.5

18979	TMP-1069	13.9	37.4	36.3	46.9	17.0	37.5	189.0	1.8
97610	TMP-2170	6.2	14.8	17.9	17.9	6.0	18.4	81.2	2.0
	CDC Bethune	12.3	36.9	44.0	45.0	14.4	47.1	199.7	2.1
33388	PGR-5039	9.1	24.4	28.2	32.4	10.4	31.4	135.8	2.1
101610	TMP-13685	11.1	36.3	28.9	30.5	14.3	31.1	152.2	1.5
98704	TMP-8230	7.5	22.4	29.3	39.3	17.6	33.1	149.2	2.1
96962	TMP-8451	9.9	29.7	42.9	44.0	11.0	40.4	177.9	2.5
	CDC Bethune	11.4	34.1	39.8	41.7	13.3	43.6	183.9	2.1
97670	TMP 7706-13	8.5	26.2	29.1	29.0	10.6	30.3	133.7	2.0
97953	TMP-2386	10.1	29.9	34.2	37.3	12.6	34.4	158.5	2.0
97083	TMP 2724-10	13.2	37.5	37.3	39.2	14.8	39.2	181.2	1.8
19158	TMP-1508	9.1	27.8	34.1	39.2	10.5	37.3	157.9	2.3
	CDC Bethune	10.9	33.5	37.5	40.7	13.2	40.4	176.2	2.1
101099	TMP-1838	no seed material							
98826	TMP-7516	14.4	44.0	43.3	42.8	17.5	41.7	203.6	1.7
98954	TMP-2261	7.7	21.0	25.3	28.6	9.7	28.7	120.9	2.2
	TMP-10097	no seed material							
	CDC Bethune	14.9	47.0	45.7	49.8	18.8	46.2	222.3	1.8
100797	TMP 8360-6	9.6	29.8	43.6	53.2	11.4	43.7	191.2	2.8
	TMP-2940	no seed material							
33386	PGR-5037	9.2	25.8	31.7	33.1	11.0	33.3	144.1	2.1
	TMP-2677	no seed material							
	CDC Bethune	14.3	41.7	46.1	47.7	19.6	47.9	217.4	1.9
100863	TMP-1435	10.6	33.3	35.0	44.0	12.3	37.1	172.2	2.1
	TMP 14331	15.2	48.1	48.5	51.4	23.2	40.5	226.9	1.6
101096	TMP-1835	10.9	33.3	33.9	30.3	13.5	32.1	153.9	1.7
101471	TMP-9922	6.0	20.0	36.1	40.7	8.8	36.6	148.1	3.3
	CDC Bethune	13.7	41.6	43.3	43.6	20.3	43.1	205.6	1.7
30860	PGR-1725	7.5	24.3	29.5	32.2	9.6	29.2	132.3	2.2
101386	TMP-13186	7.1	21.2	29.3	31.6	10.1	32.7	132.0	2.4
98946	TMP-2242	8.5	26.9	29.0	33.5	9.9	30.6	138.3	2.1
	TMP-8020	no seed material							
	CDC Bethune	12.5	42.5	41.3	39.4	18.9	39.8	194.4	1.6
98634	TMP-8158	no seed material							
101338	TMP-13137	7.8	23.7	33.4	34.0	9.6	33.1	141.5	2.4
101241	TMP-10817	11.3	35.0	29.3	28.5	13.0	29.6	146.6	1.5
98752	TMP-8284	10.6	32.4	38.9	37.9	12.6	36.9	169.3	2.0
	CDC Bethune	10.7	27.7	38.0	40.5	12.1	42.0	170.9	2.4
97642	TMP-7687	12.2	34.9	34.3	36.5	14.0	34.8	166.6	1.7
100629	TMP-11172	8.6	25.3	53.0	47.7	10.2	51.1	195.8	3.4
97458	TMP-2322	8.5	28.2	31.3	33.5	13.3	30.4	145.4	1.9
98734	TMP-8268	10.4	31.9	37.4	37.1	12.6	37.2	166.6	2.0
	CDC Bethune	9.0	26.5	35.0	39.1	7.1	37.7	154.5	2.6

33390	PGR-5041				no seed material				
97453	TMP-7530	15.5	45.2	38.4	43.4	15.8	38.2	196.5	1.6
101486	TMP-9935	9.0	29.3	28.7	28.2	11.7	28.2	135.0	1.7
19005	TMP-1466	10.4	31.1	27.2	33.6	13.1	30.2	145.7	1.7
	CDC Bethune	10.0	32.0	29.6	31.5	12.9	31.4	147.4	1.7
18989	TMP-1158	10.1	31.1	35.6	37.3	11.3	28.4	153.8	1.9
98276	TMP-2536				no seed material				
98794	TMP-8329	11.5	34.2	34.9	38.1	13.0	35.8	167.5	1.9
101385	TMP-13185	9.7	30.6	34.8	35.1	12.6	35.6	158.4	2.0
	CDC Bethune	14.4	48.2	43.5	43.0	18.8	44.9	212.8	1.6
100807	TMP-1368	5.5	16.6	25.9	26.5	7.5	25.2	107.1	2.6
101598	TMP-10126	6.6	20.5	29.6	31.9	9.0	28.4	126.1	2.5
101325	TMP-13124	9.8	28.9	32.2	31.5	13.2	31.0	146.5	1.8
98984	TMP-8172	10.7	34.8	38.9	38.9	14.4	38.1	175.8	1.9
	CDC Bethune	11.5	34.6	36.7	37.2	14.9	39.2	174.1	1.9
32546	PGR-4048				no seed material				
101419	TMP-1922	10.1	32.1	30.5	29.7	13.4	29.6	145.4	1.6
98903	TMP 2202-8	11.5	32.5	39.8	39.7	14.6	38.6	176.6	2.0
101595	TMP-10020	11.0	24.7	40.8	40.0	26.9	40.9	184.2	1.9
	CDC Bethune	12.0	38.3	38.5	43.7	15.4	42.5	190.3	1.9
96845	TMP-8373	7.5	22.8	33.5	40.2	9.2	36.5	149.7	2.8
	TMP-9981				no seed material				
97407	TMP-2988	10.0	36.5	38.7	35.7	14.6	34.3	169.9	1.8
101511	TMP-9953				no seed material				
	CDC Bethune	14.0	41.9	40.6	42.6	21.2	41.7	201.9	1.6
101405	TMP-13212	11.1	36.6	28.1	29.2	13.7	27.8	146.6	1.4
97649	TMP-7694	9.5	33.6	35.3	31.3	15.2	33.0	157.8	1.7
97444	TMP-7521	12.3	40.5	38.8	37.7	16.6	37.0	183.0	1.6
101463	TMP-10098	8.6	25.8	33.2	41.8	10.9	39.1	159.4	2.5
	CDC Bethune	10.5	33.1	34.4	35.8	14.0	35.7	163.6	1.8
97639	TMP 7684-11	10.1	30.0	38.8	41.3	12.7	36.8	169.8	2.2
	TMP-2894				no seed material				
100828	TMP-1396	9.3	26.2	37.8	33.9	11.6	36.5	155.3	2.3
98869	TMP-8482	9.6	28.5	34.4	35.3	12.1	33.3	153.2	2.1
	CDC Bethune	10.3	32.3	30.0	37.4	11.9	33.4	155.2	1.8
97520	TMP-7590	10.1	33.0	38.4	37.6	12.3	38.8	170.3	2.1
	Macbeth	13.2	41.1	40.1	42.4	17.8	43.2	197.8	1.7
101367	TMP-13166	11.3	38.0	33.3	33.1	13.0	31.6	160.4	1.6
	Prairie Blue	11.8	41.7	33.2	32.3	19.1	29.1	167.2	1.3
	CDC Bethune	13.1	40.2	43.5	52.9	17.7	46.0	213.5	2.0
96992	TMP-2088	10.8	37.7	38.4	36.2	16.0	17.3	156.2	1.4
98541	TMP-2934	8.7	26.0	37.4	37.2	12.9	36.2	158.4	2.3
100864	TMP-1437	6.2	24.8	32.9	33.4	16.1	30.5	143.8	2.1

33400	PGR-5051	16.1	43.4	36.6	41.6	15.6	38.2	191.4	1.6
	CDC Bethune	9.5	29.8	29.6	29.8	11.7	31.2	141.6	1.8
97958	TMP-2390	11.9	38.4	33.0	36.1	15.9	32.5	167.9	1.5
97396	TMP-2978	9.1	29.1	33.4	35.6	13.8	34.1	154.9	2.0
33397	PGR-5048	7.3	25.6	32.9	34.2	13.5	32.9	146.3	2.2
97287	TMP-2610	10.6	34.0	34.3	34.3	15.8	33.7	162.8	1.7
	CDC Bethune	13.8	42.5	39.7	38.8	16.7	39.0	190.4	1.6
98279	TMP-10221	14.0	37.4	33.8	36.3	15.7	34.3	171.5	1.6
98807	TMP-8340	13.1	39.8	47.8	51.0	26.4	45.6	223.7	1.8
101286	TMP-10873	7.2	18.6	29.5	31.2	8.8	30.5	125.7	2.6
97890	TMP-8382	7.5	19.4	32.4	34.9	12.5	34.7	141.4	2.6
	CDC Bethune	9.8	29.1	33.0	34.1	12.7	36.0	154.7	2.0
98150	TMP-2192	10.7	30.6	0.0	33.8	13.1	0.0	88.1	0.6
101366	TMP-13165	7.7	22.0	34.7	34.4	10.0	35.3	144.1	2.6
97489	TMP-7559	10.3	24.4	35.4	39.7	11.3	39.1	160.2	2.5
97430	TMP-2998	6.5	19.4	34.1	35.0	21.7	34.9	151.5	2.2
	CDC Bethune	11.6	35.2	39.5	41.7	13.8	41.3	183.2	2.0
100827	TMP-1395	5.8	13.3	39.8	46.5	17.3	42.5	165.1	3.5
101572	TMP-10118	6.7	18.5	29.1	35.1	15.6	31.0	136.1	2.3
	TMP-1423				no seed material				
101016	TMP-1719	5.8	17.1	25.2	30.1	7.0	27.3	112.6	2.8
	CDC Bethune	13.4	42.8	43.2	43.6	21.6	42.0	206.4	1.7
	Shape	10.2	29.6	32.5	40.7	13.2	38.3	164.4	2.1
97366	TMP-8641	10.2	31.2	33.3	41.6	12.7	37.6	166.7	2.1
101421	TMP-1924				no seed material				
100838	TMP-1409				no seed material				
	CDC Bethune	11.4	31.1	40.2	42.9	13.3	44.4	183.3	2.3
97350	TMP-8235	8.3	25.1	36.0	36.4	10.5	36.1	152.3	2.5
100547	PGR-14271	9.1	24.7	34.3	35.9	10.5	37.4	152.0	2.4
	Prairie Thunder	11.9	31.8	44.4	49.2	12.7	48.0	198.0	2.5
97584	TMP-7646	4.6	12.3	14.7	16.2	5.4	15.5	68.6	2.1
	CDC Bethune	9.6	29.1	38.4	39.5	11.0	40.7	168.3	2.4
97728	TMP-7760	6.2	17.8	26.0	33.0	7.2	29.2	119.5	2.8
98193	TMP-2530	9.8	30.4	33.6	34.0	13.4	33.7	155.0	1.9
101137	TMP-8784	8.5	24.6	47.7	59.3	9.9	51.7	201.7	3.7
101397	TMP-13197	10.6	26.7	35.2	38.3	12.0	38.2	161.1	2.3
	CDC Bethune	11.3	36.1	38.4	44.2	14.3	43.3	187.5	2.0
97586	TMP-7648	6.2	17.7	33.9	37.0	7.3	35.9	137.9	3.4
101496	TMP-13674	8.6	22.8	29.2	31.5	10.6	32.2	134.8	2.2
97533	TMP 7619-2	11.3	28.8	38.3	38.7	11.6	38.5	167.1	2.2
97470	TMP-10230	9.3	27.6	30.3	33.1	11.5	32.4	144.2	2.0
	CDC Bethune	12.0	36.0	38.2	39.7	13.4	39.4	178.8	1.9

19004	TMP-1311	7.6	21.0	24.5	27.5	9.0	27.3	117.0	2.1
	TMP-2862				no seed material				
97671	TMP-2173	8.6	22.7	37.0	39.2	9.2	37.5	154.2	2.8
98176	TMP-8479	8.7	20.9	34.1	34.7	11.7	35.6	145.6	2.5
	CDC Bethune	11.0	31.8	37.7	39.0	12.4	39.2	171.1	2.1
33385	PGR-5036	11.1	31.7	32.2	36.8	12.5	34.7	158.9	1.9
101114	TMP-1853	10.7	30.0	39.2	38.2	12.2	42.0	172.3	2.3
98467	TMP-2923				no seed material				
98303	TMP-2275	8.9	26.4	36.9	37.9	11.0	37.8	158.8	2.4
	CDC Bethune	14.0	42.5	41.0	43.7	17.9	42.9	202.1	1.7
97092	TMP-8556	7.9	20.8	38.1	37.9	9.1	40.4	154.2	3.1
100881	TMP-1455	8.6	22.9	32.4	33.4	11.8	32.8	141.8	2.3
98056	TMP-2181	8.1	25.3	1.2	27.5	9.3	0.0	71.4	0.7
97072	TMP 2713-4	8.9	24.3	32.8	31.9	95.0	35.0	227.9	0.8
	CDC Bethune	11.8	35.8	34.6	35.1	15.2	35.7	168.2	1.7
	TMP-1595				no seed material				
40081	PGR-13075	10.0	26.4	41.1	40.8	10.8	43.4	172.6	2.7
19001	TMP-1171	9.7	26.8	36.6	37.2	11.2	38.7	160.2	2.4
97487	TMP-7557	11.1	34.5	35.9	39.1	15.0	37.5	173.1	1.9
	CDC Bethune	11.6	35.5	35.4	36.3	14.9	36.4	170.3	1.7
98708	TMP-8233	7.9	22.7	30.0	33.9	15.7	31.0	141.1	2.1
98192	TMP-8006	6.7	22.1	27.9	26.5	10.3	27.6	121.1	2.1
98542	TMP-2622	10.7	25.4	42.2	42.3	11.2	44.3	176.1	2.7
97377	TMP-2968	7.4	21.8	37.8	40.4	9.0	41.6	158.0	3.1
	CDC Bethune	12.0	36.6	38.9	46.4	15.3	44.2	193.6	2.0
101230	TMP-10803	7.9	22.6	27.4	26.9	10.3	27.1	122.2	2.0
19160	TMP-1384	6.0	16.6	32.5	40.1	11.4	37.0	143.6	3.2
98639	TMP-8163	7.7	25.2	34.1	36.4	10.8	45.9	160.2	2.7
100883	TMP-1457	8.6	23.7	36.0	37.2	19.2	38.4	163.0	2.2
	CDC Bethune	12.0	37.2	34.5	33.9	13.8	36.4	167.6	1.7
97056	TMP-2697	6.5	17.5	35.2	35.1	7.7	36.4	138.5	3.4
101413	TMP-1174	9.9	27.2	31.6	34.5	11.8	34.7	149.7	2.1
98806	TMP-8339	6.7	19.9	25.9	28.4	4.3	29.8	115.0	2.7
101332	TMP-13131	9.6	23.6	31.9	34.7	13.4	34.8	148.0	2.2
	CDC Bethune	11.6	31.0	39.0	42.1	13.1	41.9	178.6	2.2
98733	TMP 8267-8	8.6	25.7	33.4	34.9	10.5	35.5	148.6	2.3
101039	TMP-1778	10.3	29.9	35.1	35.1	12.8	37.8	161.0	2.0
101416	TMP-1919	8.4	23.5	28.8	29.4	10.1	30.3	130.5	2.1
97749	TMP-7780	7.0	22.1	35.3	36.7	8.5	37.9	147.4	2.9
	CDC Bethune	15.9	46.7	44.7	46.1	19.1	46.0	218.5	1.7
98397	TMP-2844				no seed material				
97718	TMP-7751	5.7	17.0	29.2	30.4	14.6	31.7	128.6	2.4
98753	TMP-8285	13.5	32.4	34.4	38.6	13.2	35.0	167.1	1.8

	TMP-2953				no seed material				
	CDC Bethune	13.8	43.7	39.9	43.2	19.4	40.6	200.5	1.6
97139	TMP-2772				no seed material				
97587	TMP-7649	8.0	23.1	31.8	31.5	10.1	31.3	135.8	2.3
33992	PGR-5772	10.3	30.0	37.1	42.0	11.9	40.3	171.4	2.3
97403	TMP-2984	9.9	28.6	33.7	36.5	11.7	36.7	157.1	2.1
	CDC Bethune	13.9	45.9	43.4	41.6	18.2	41.3	204.4	1.6
98505	TMP-8598	6.0	19.6	25.2	25.7	8.4	25.5	110.4	2.2
	CDC Sorrel	18.1	51.9	33.3	37.0	37.8	35.7	213.8	1.0
97430	TMP 2998-9	5.9	18.2	32.4	32.8	20.8	33.3	143.4	2.2
97881	TMP-7880	9.6	27.1	28.1	29.9	10.7	30.7	136.1	1.9
	CDC Bethune	10.0	30.8	35.9	37.9	12.4	38.6	165.7	2.1
101127	TMP-1866	12.7	38.9	36.1	36.2	17.9	36.0	177.8	1.6
19157	TMP-1436	11.3	34.4	30.8	36.1	16.0	31.1	159.7	1.6
97129	TMP 2124-4	6.5	18.5	32.7	31.8	11.6	33.2	134.3	2.7
98037	TMP 2467-8	9.2	29.7	33.6	32.0	11.3	34.8	150.6	2.0
	CDC Bethune	11.3	31.9	38.5	39.9	15.0	41.0	177.7	2.1
97529	TMP-7597				no seed material				
98854	TMP-10222	9.8	30.4	36.3	36.9	13.1	38.7	165.1	2.1
101379	TMP-13179	13.1	38.9	40.2	44.4	15.1	41.5	193.2	1.9
18994	TMP-1163	8.9	28.2	29.1	32.6	13.4	30.5	142.8	1.8
	CDC Bethune	11.1	33.0	37.4	38.4	14.4	40.4	174.8	2.0
98157	TMP-7973	8.6	26.1	47.4	49.1	10.2	46.1	187.5	3.2
97584	TMP 7646-7	11.8	36.1	32.3	36.3	15.6	33.8	166.0	1.6
101392	TMP-13192				no seed material				
97571	TMP-7636	10.1	32.1	41.5	40.2	12.7	38.9	175.6	2.2
	CDC Bethune	10.3	29.5	30.8	31.5	13.2	32.9	148.1	1.8
97050	TMP-2695				no seed material				
97064	TMP-2705				no seed material				
98475	TMP-2279	5.0	15.2	21.8	22.3	12.1	22.2	98.6	2.1
101154	TMP-9786				no seed material				
	CDC Bethune	10.4	32.0	30.5	29.4	12.1	30.1	144.5	1.7
97129	TMP-2124	7.6	18.5	34.1	32.6	8.2	32.9	133.8	2.9
100848	TMP-1419	6.7	18.9	29.7	31.9	8.2	32.8	128.3	2.8
	TMP-2960				no seed material				
100841	TMP-1412	11.7	34.1	36.6	41.8	15.8	38.6	178.5	1.9
	CDC Bethune	12.6	39.6	40.6	45.3	15.1	43.1	196.4	1.9
101466	TMP-10135				no seed material				
97153	TMP-8574	9.4	25.5	38.4	39.9	13.2	40.3	166.7	2.5
100785	TMP-11332	8.5	25.2	25.0	28.1	10.9	28.9	126.6	1.8
98689	TMP 8216-5	3.9	11.5	27.5	28.0	5.0	27.7	103.6	4.1
	CDC Bethune	14.9	46.1	45.5	44.6	16.3	48.4	215.8	1.8
98135	TMP-2671				no seed material				

97740	TMP-7771	7.0	20.4	27.6	30.0	8.2	30.1	123.2	2.5
98072	TMP-2187	6.9	20.4	24.4	25.5	5.5	25.2	107.8	2.3
97393	TMP-2629	5.4	15.0	26.1	31.4	7.0	28.2	113.1	3.1
	CDC Bethune	11.0	34.2	42.6	45.1	13.7	46.5	193.1	2.3
97484	TMP-7554	9.7	27.2	33.3	34.6	12.8	35.6	153.2	2.1
97341	TMP 8152-12	10.5	31.2	29.2	30.6	15.5	32.2	149.1	1.6
98027	TMP-2457	8.6	26.9	28.4	27.0	12.0	28.5	131.4	1.8
35791	PGR-8233				no seed material				
	CDC Bethune	14.4	40.8	45.7	48.4	18.4	47.9	215.6	1.9
	TMP-9982				no seed material				
98363	TMP-2810				no seed material				
101559	TMP-10091	7.2	22.1	22.3	22.8	8.6	23.9	106.9	1.8
33389	PGR-5040	7.3	22.5	26.4	29.6	9.5	30.2	125.4	2.2
	CDC Bethune	11.9	35.3	37.6	39.3	18.1	37.8	180.0	1.8
98263	TMP-2534	6.2	15.4	37.4	35.9	7.5	38.5	140.9	3.8
97312	TMP-2948	5.4	16.1	26.5	26.9	6.2	28.4	109.6	3.0
	TMP-2280				no seed material				
97214	TMP-2564				no seed material				
	CDC Bethune	10.8	32.3	38.9	41.3	13.2	41.5	178.0	2.2
101404	TMP-13211	10.2	30.0	26.3	28.7	13.6	27.1	135.9	1.5
98742	TMP 8274-8	8.8	24.7	27.5	29.9	11.2	28.6	130.7	1.9
98278	TMP-2538	8.3	25.5	32.5	31.4	11.3	32.9	142.0	2.1
98165	TMP-7982	7.6	21.2	30.8	31.3	13.1	30.3	134.3	2.2
	CDC Bethune	12.6	45.2	37.5	38.3	16.7	37.2	187.5	1.5
19003	TMP-1310	8.8	25.7	32.0	33.9	10.5	33.6	144.4	2.2
101396	TMP-13196	8.4	24.7	28.2	27.3	10.3	28.4	127.3	1.9
97633	TMP-7678	4.3	14.5	26.0	27.8	6.3	28.9	107.8	3.3
37286	PGR-10014	7.7	22.8	31.9	34.1	10.3	35.3	142.2	2.5
	CDC Bethune	13.3	38.1	39.7	41.1	15.7	39.0	187.0	1.8
	Prairie Grande	10.3	29.9	29.1	31.3	12.0	30.5	143.0	1.7
98821	TMP-8123	6.5	19.2	21.7	23.5	7.6	23.7	102.2	2.1
96958	TMP 2650-14	7.4	21.0	39.8	39.5	11.7	40.0	159.3	3.0
101482	TMP-10083	6.3	19.4	30.7	33.5	8.4	34.0	132.3	2.9
	CDC Bethune	13.8	42.6	38.5	40.1	16.7	39.5	191.3	1.6
101403	TMP-13210	9.4	29.0	23.9	24.2	12.6	25.1	124.2	1.4
18982	TMP-1151	9.4	29.2	37.1	36.5	12.3	37.8	162.2	2.2
98934	TMP-2230	7.5	22.7	25.0	27.9	9.5	27.8	120.4	2.0
98056	TMP 2181-15	9.9	28.8	13.2	35.7	13.2	13.4	114.2	1.2
	CDC Bethune	14.0	40.3	39.3	42.3	18.0	40.3	194.2	1.7
97967	TMP-2399	9.3	28.1	28.9	28.5	12.0	28.7	135.5	1.7
98982	TMP-2922				no seed material				
	Hanley	19.8	59.7	52.6	58.5	23.1	49.4	263.0	1.6
98239	TMP-8535	10.0	30.6	27.7	31.2	13.1	30.0	142.7	1.7

	CDC Bethune	12.1	36.8	39.1	44.8	13.8	40.9	187.5	2.0
	TMP-2895				no seed material				
100837	TMP-1408	10.2	39.1	36.4	37.1	11.6	37.4	171.8	1.8
96974	TMP 2652-15				no seed material				
101055	TMP-1794	9.3	26.6	24.7	25.3	11.1	25.9	122.9	1.6
	CDC Bethune	12.5	38.6	35.8	39.0	14.5	36.5	176.7	1.7
	TMP 17618	12.0	36.1	32.9	38.2	15.2	36.7	171.1	1.7
98037	TMP-2467	7.3	21.9	28.5	27.9	8.8	29.7	124.1	2.3
100929	TMP-1614	9.6	30.5	28.5	28.6	13.6	28.9	139.7	1.6
	TMP-13163				no seed material				
	CDC Bethune	11.0	35.7	39.4	45.7	13.6	42.2	187.4	2.1
98923	TMP 2222-4	8.2	26.6	19.5	19.7	11.3	20.4	105.7	1.3
101118	TMP-1857				no seed material				
101378	TMP-13178	9.6	30.2	31.9	32.8	13.0	33.4	151.0	1.9
	TMP 2679-15				no seed material				
	CDC Bethune	13.3	42.9	42.8	43.4	21.1	44.4	207.8	1.7
101472	TMP-10082				no seed material				
	Lightning	10.2	30.5	34.3	38.3	12.2	36.9	162.5	2.1
98644	TMP 8168-3				no seed material				
97873	TMP-10239	9.5	30.5	28.4	30.4	12.9	29.3	140.9	1.7
	CDC Bethune	10.6	31.7	37.5	36.1	15.5	34.4	165.7	1.9
101448	TMP-10030				no seed material				
18997	TMP-1167				no seed material				
97871	TMP 2177-7	9.1	28.6	28.1	29.2	12.5	28.6	136.2	1.7
18987	TMP-1156				no seed material				
	CDC Bethune	11.3	34.7	38.8	41.0	14.1	43.2	183.2	2.0
101301	TMP-10889				no seed material				
98613	TMP-2963				no seed material				
97604	TMP-8375	10.1	30.7	32.2	32.3	13.0	32.7	150.9	1.8
100928	TMP-1613	6.9	22.3	31.7	31.4	17.8	31.6	141.7	2.0
	CDC Bethune	9.6	30.2	35.0	38.3	12.6	40.2	166.0	2.2
97406	TMP 2987-14	10.8	32.3	31.9	33.8	13.2	32.2	154.1	1.7
100678	TMP-11224				no seed material				
100895	TMP-1580	10.7	33.1	34.4	34.5	13.8	34.3	160.8	1.8
101331	TMP-13130	10.1	29.0	41.3	42.5	12.0	42.0	176.9	2.5
	CDC Bethune	16.0	50.1	45.6	46.8	21.4	45.6	225.4	1.6
	Hanley	10.2	29.9	34.5	38.9	14.2	38.4	166.0	2.1
	Macbeth	12.6	38.5	39.1	48.2	14.6	45.1	198.1	2.0
	TMP-2795				no seed material				
98109	TMP-2659				no seed material				
	CDC Bethune	12.5	40.1	39.1	39.0	15.9	39.6	186.2	1.7
101265	TMP-10847	9.2	29.0	31.2	34.9	12.0	32.4	148.6	2.0
98812	TMP-8345	9.1	24.7	23.6	32.1	10.3	28.1	128.0	1.9

	TMP-8017				no seed material				
100885	TMP-1460	6.8	21.4	31.3	35.0	33.5	31.0	159.0	1.6
	CDC Bethune	13.7	43.7	42.2	42.9	17.1	44.1	203.7	1.7
	TMP-10021				no seed material				
97321	TMP-8126	9.1	28.0	36.5	36.9	11.9	34.1	156.6	2.2
	TMP-9984				no seed material				
101136	TMP-1876	11.6	36.3	33.3	32.1	15.1	33.9	162.4	1.6
	CDC Bethune	12.9	40.8	37.7	43.8	17.9	40.3	193.4	1.7
52732	PGR-27314	10.4	28.9	35.8	40.1	11.6	38.2	164.9	2.2
97907	TMP-7899	11.6	30.9	38.2	43.4	12.6	40.9	177.6	2.2
101208	TMP-9853	9.0	26.5	29.5	35.9	11.2	33.7	145.8	2.1
101116	TMP-1855	10.1	29.7	27.9	28.7	12.0	29.1	137.5	1.7
	CDC Bethune	11.0	34.1	37.0	38.2	13.5	40.3	174.0	2.0
98286	TMP-2269	10.2	28.2	34.6	34.4	13.8	33.6	154.6	2.0
97961	TMP-2393	10.3	31.0	28.3	30.3	12.4	28.9	141.2	1.6
101237	TMP-10812	11.3	32.6	36.0	37.1	14.0	36.2	167.3	1.9
101402	TMP-13203	9.3	25.7	32.6	38.3	11.2	35.3	152.4	2.3
	CDC Bethune	12.6	38.8	40.2	38.1	16.0	38.6	184.3	1.7
98263	TMP 2534-5	7.0	19.8	33.8	35.3	8.3	34.6	138.7	3.0
97096	TMP-2756				no seed material				
97300	TMP-8073	10.4	31.9	36.4	33.6	16.2	35.5	164.1	1.8
100851	TMP-1422	8.9	28.3	41.9	47.2	34.1	47.2	207.6	1.9
	CDC Bethune	10.3	30.8	29.5	30.7	12.2	31.6	145.1	1.7
32542	PGR-4044	11.6	35.6	35.3	37.1	17.1	36.3	173.0	1.7
	TMP-2924				no seed material				
33393	PGR-5044	19.3	30.6	34.1	23.9	7.9	34.5	150.3	1.6
100939	TMP-1507	9.4	30.3	39.4	42.3	11.9	39.0	172.2	2.3
	CDC Bethune	11.9	38.8	40.6	42.6	19.8	39.9	193.6	1.7
97238	TMP-2932	10.7	34.7	36.2	35.5	18.6	35.5	171.1	1.7
101296	TMP-10884	10.0	29.5	31.6	30.6	12.2	32.5	146.4	1.8
97503	TMP 7573-6	11.1	32.3	37.2	38.7	15.4	37.2	172.0	1.9
98100	TMP-2508	8.3	28.8	43.9	41.3	34.9	38.3	195.5	1.7
	CDC Bethune	9.9	28.0	34.5	35.5	12.1	36.9	156.8	2.1
	Macbeth	19.6	55.6	56.7	62.7	22.4	51.7	268.6	1.8
	Hanley	11.0	34.0	32.6	42.1	13.6	36.5	169.9	1.9
	TMP-2817				no seed material				
101299	TMP-10887	12.1	34.2	35.2	35.9	17.4	34.3	169.1	1.7
	CDC Bethune	11.1	30.8	36.5	38.8	12.3	38.0	167.5	2.1
101375	TMP-13175				no seed material				
19017	TMP-8663	9.9	30.4	33.1	34.0	14.5	34.6	156.5	1.9
	TMP-13194				no seed material				
97616	TMP-7670	12.9	40.4	34.5	35.9	17.4	33.8	175.0	1.5
	CDC Bethune	11.3	32.1	38.6	40.8	13.8	42.1	178.7	2.1

97424	TMP-2147				no seed material				
	TMP-2160				no seed material				
101279	TMP-10862	16.2	34.8	37.5	43.6	12.9	39.8	184.8	1.9
101308	TMP-13107	10.1	33.4	36.8	38.8	14.9	36.7	170.6	1.9
	CDC Bethune	17.9	51.3	42.1	46.9	20.3	43.7	222.2	1.5
98364	TMP-2811				no seed material				
101240	TMP-10816	10.3	31.1	28.4	30.7	13.6	28.8	142.9	1.6
101327	TMP-13126	8.1	24.1	27.8	26.1	12.3	27.3	125.8	1.8
18981	TMP-1097	9.9	28.8	36.6	37.1	13.2	35.6	161.3	2.1
	CDC Bethune	12.7	37.0	40.5	42.9	13.9	42.6	189.6	2.0
101132	TMP-1871	9.2	28.2	38.0	38.2	17.3	36.6	167.5	2.1
98535	TMP-8643	9.5	30.1	37.0	36.8	15.7	34.9	164.0	2.0
18986	TMP-1155	13.1	44.2	41.0	40.8	18.0	40.7	197.7	1.6
101600	TMP-10024	11.6	32.2	33.1	39.2	11.5	35.4	162.9	1.9
	CDC Bethune	10.8	35.0	37.7	43.0	14.3	41.8	182.5	2.0
101401	TMP-13202	9.7	27.2	28.3	31.8	12.0	29.4	138.4	1.8
97004	TMP-8464	7.0	19.9	29.0	29.6	9.9	28.6	124.0	2.4
97679	TMP 7714-14	7.5	23.6	35.4	35.6	12.4	35.0	149.5	2.4
	TMP 2679-9				no seed material				
	CDC Bethune	11.4	35.3	40.6	42.2	14.4	42.9	186.7	2.1
101119	TMP-1858	10.5	34.3	37.4	36.2	16.9	36.8	172.1	1.8
97679	TMP-7714	7.9	24.7	37.1	37.5	13.4	37.7	158.2	2.4
101310	TMP-13109	6.3	19.2	29.6	32.6	17.5	31.3	136.6	2.2
101026	TMP-1729	7.4	24.2	34.5	33.7	12.6	35.7	148.2	2.3
	CDC Bethune	13.7	41.3	42.3	45.3	16.6	43.2	202.4	1.8
96988	TMP-2084	7.7	25.1	40.5	42.7	10.6	41.0	167.6	2.9
100797	TMP 8360-8	7.2	23.0	39.2	44.9	9.9	40.9	165.2	3.1
18998	TMP-1168	10.8	37.9	44.3	43.3	16.2	43.5	196.0	2.0
18988	TMP-1157	11.6	37.0	47.4	50.2	17.3	49.1	212.6	2.2
	CDC Bethune	10.7	32.6	38.3	39.9	13.3	40.2	174.9	2.1
98254	TMP-8018	6.5	21.2	34.8	35.9	9.5	37.6	145.5	2.9
18973	TMP-605	12.6	41.3	48.6	51.5	17.1	51.6	222.6	2.1
97392	TMP-2975	8.9	28.7	30.8	32.0	11.2	33.7	145.4	2.0
101348	TMP-13147	13.1	40.8	41.1	47.5	15.5	42.1	200.2	1.9
	CDC Bethune	14.3	40.8	40.1	44.8	16.3	41.8	198.1	1.8
100674	TMP-11220	10.2	33.0	35.5	39.9	11.6	38.7	169.0	2.1
	TMP-9941				no seed material				

Data are presented as mean value from two replications

Table D.2 Profile of CLs (mAU) in oxidized methanol extract of flax accessions of core collection grown at Morden, MB in 2009

CN	TMP	1-Mso,3-Mso-CLF	1-Mso,3-Mso-CLG	1-Mso-CLB	1-Mso-CLE	1-Mso-CLD	CLA	Total	Ratio
101373	TMP-13173	7.4	25.9	34.5	31.1	9.0	32.7	140.5	2.3
98683	TMP-8209	12.7	35.6	34.9	35.3	11.9	38.5	169.0	1.8
	CDC Bethune				no seed material				
	TMP 2681-1				no seed material				
100884	TMP-1459	12.5	24.5	41.2	43.0	8.4	39.9	169.6	2.7
101382	TMP-13182	11.7	30.9	29.4	30.7	10.3	32.2	145.2	1.7
98741	TMP 8273-2	8.5	24.9	42.4	42.4	9.7	45.3	173.1	3.0
	CDC Bethune	8.4	23.1	26.4	37.8	124.9	27.4	248.0	0.6
30861	PGR-1726	19.0	37.9	21.5	39.2	11.9	35.7	165.3	1.4
97402	TMP 2983-13	14.2	42.3	43.1	44.8	15.4	44.2	204.1	1.8
97404	TMP-2985	15.1	44.9	31.9	40.1	17.7	39.1	188.9	1.4
98710	TMP 8238-10	12.2	38.6	44.2	48.3	17.4	46.2	207.0	2.0
	CDC Bethune	8.6	28.4	38.5	40.3	11.8	38.8	166.5	2.4
	TMP-9996				no seed material				
98566	TMP 2945-7	6.1	19.0	32.2	28.8	7.7	27.9	121.6	2.7
96846	TMP-8374	9.2	22.1	30.2	30.3	7.9	32.3	132.1	2.4
98012	TMP-2443	9.3	29.6	37.0	36.7	33.4	35.0	181.0	1.5
	CDC Bethune	9.8	31.1	38.5	39.0	12.4	37.6	168.4	2.2
97180	TMP-2289	8.8	35.4	37.6	40.0	17.7	38.0	177.6	1.9
97483	TMP 7553-6	10.0	32.4	37.4	38.6	14.1	37.8	170.3	2.0
18993	TMP-1162	13.1	36.0	42.7	48.1	13.4	46.3	199.6	2.2
101535	TMP-9980				no seed material				
	CDC Bethune	10.6	35.3	35.5	38.0	14.6	35.0	168.9	1.8
97768	TMP-2343	12.1	36.6	49.6	44.5	14.7	47.6	205.1	2.2
97921	TMP-7912	8.3	26.1	29.0	32.4	9.9	29.4	135.1	2.0
98231	TMP-8533	7.8	21.2	35.3	35.9	9.9	33.1	143.2	2.7
98440	TMP-2887	11.7	38.5	31.9	32.5	15.5	31.2	161.3	1.5
	CDC Bethune	10.8	31.0	35.8	42.8	11.8	31.5	163.6	2.1
100952	TMP-1654	10.5	31.5	40.6	44.8	13.2	40.8	181.4	2.3
97404	TMP 2985-5	11.3	35.2	36.3	42.7	12.8	41.3	179.7	2.0
101407	TMP-13214	14.2	38.3	31.6	35.3	13.3	35.2	167.9	1.6
98712	TMP-8240	10.9	34.5	40.5	42.6	11.7	42.5	182.6	2.2
	CDC Bethune	11.6	36.4	42.0	44.8	14.2	46.2	195.1	2.1
97397	TMP-2979	8.6	25.0	36.2	39.8	12.3	19.5	141.5	2.1
101560	TMP-13679				no seed material				
100790	TMP-11337	12.8	38.5	58.4	53.1	16.6	54.1	233.5	2.4
101094	TMP-1833	11.9	32.9	36.7	39.4	15.5	36.5	172.8	1.9
	CDC Bethune	12.0	34.5	39.9	45.7	13.4	42.2	187.7	2.1

98039	TMP-2469	10.4	35.2	33.5	32.3	13.1	32.0	156.4	1.7
101052	TMP-1791	9.4	25.4	39.0	37.2	10.6	38.7	160.4	2.5
100805	TMP-1365	9.9	26.3	35.7	41.6	10.0	39.2	162.8	2.5
98767	TMP-8301	8.7	27.7	31.1	37.4	12.8	32.7	150.3	2.1
	CDC Bethune	12.1	38.1	42.0	44.4	15.1	42.2	193.8	2.0
101329	TMP-13128	11.7	36.0	33.2	34.6	14.1	31.1	160.8	1.6
97531	TMP-2161	13.5	38.2	47.7	47.0	15.5	44.4	206.3	2.1
98566	TMP-2945	6.2	18.3	35.3	33.9	7.7	34.3	135.8	3.2
	TMP 2945-4				no seed material				
	CDC Bethune	12.4	37.9	40.7	44.8	15.1	42.8	193.7	2.0
98926	TMP 2224-10	12.5	34.7	32.6	34.7	15.1	34.0	163.5	1.6
96911	TMP-8409	7.4	24.4	39.1	40.3	9.8	37.2	158.1	2.8
97351	TMP-8236	11.5	31.3	40.8	41.5	12.5	42.8	180.3	2.3
	TMP-1777				no seed material				
	CDC Bethune	12.4	38.7	32.6	38.0	14.8	33.2	169.8	1.6
98829	TMP-2156	12.8	40.5	40.0	41.5	17.1	41.8	193.7	1.8
96991	TMP-2087	7.5	20.8	35.9	39.6	8.5	40.2	152.4	3.1
98014	TMP-2445	10.6	30.9	38.9	39.9	14.9	39.9	175.2	2.1
19159	TMP-1354	13.4	40.0	49.2	45.6	17.0	46.8	211.9	2.0
	CDC Bethune	12.4	36.2	41.6	46.5	14.2	45.8	196.7	2.1
	Hanley	11.7	34.6	39.9	43.7	13.6	42.6	186.1	2.1
	Macbeth				no seed material				
97463	TMP-7537				no seed material				
	Macbeth	13.4	45.5	47.0	48.3	15.7	45.0	214.8	1.9
	CDC Bethune	12.5	33.6	41.8	47.1	91.6	43.4	270.0	1.0
	Hanley	12.0	42.5	43.6	42.0	13.9	40.3	194.4	1.8
97665	TMP 7702-5	13.0	40.5	44.7	47.6	17.5	23.5	186.9	1.6
97103	TMP-8560	12.9	38.6	35.0	31.5	13.3	34.1	165.4	1.6
101307	TMP-10896	15.6	28.3	46.1	55.9	78.1	47.8	271.9	1.2
	CDC Bethune	12.5	39.6	38.1	38.5	15.8	41.5	186.0	1.7
	TMP-8376				no seed material				
101510	TMP-9952	8.9	26.9	48.7	48.9	10.6	52.8	196.8	3.2
97886	TMP-2361	12.9	38.4	41.1	45.3	14.9	44.5	197.1	2.0
19007	TMP-1440	7.0	21.2	29.3	31.1	11.5	30.4	130.6	2.3
	CDC Bethune	12.6	34.9	42.5	42.3	13.6	41.0	186.9	2.1
33399	PGR-5050	13.2	40.6	35.8	35.9	14.1	33.9	173.6	1.6
101298	TMP-10886	11.4	33.6	46.7	50.6	14.2	47.0	203.5	2.4
98773	TMP-8307	10.8	30.3	43.6	44.1	187.9	42.8	359.5	0.6
101115	TMP-1854	12.7	38.8	30.7	29.7	14.8	32.2	158.9	1.4
	CDC Bethune	12.6	40.2	35.9	36.9	14.3	36.9	176.8	1.6
101289	TMP-10877	12.5	39.4	35.3	35.0	14.7	33.0	170.0	1.5
100795	TMP-10250	12.4	36.2	26.8	28.4	13.6	30.0	147.3	1.4
101406	TMP-13213	12.2	35.2	43.4	44.3	14.6	46.3	196.2	2.2

98007	TMP-2438	9.0	25.8	27.2	27.9	9.6	27.5	126.8	1.9
	CDC Bethune	12.6	39.5	40.3	48.2	15.0	41.6	197.3	1.9
101469	TMP-10112	8.9	26.6	36.8	40.8	12.4	34.5	160.0	2.3
97475	TMP-7544	9.8	30.3	31.9	32.8	14.3	32.4	151.6	1.8
97613	TMP-7666	14.1	45.7	43.5	43.1	17.4	44.8	208.6	1.7
18983	TMP-1152	9.7	29.2	35.6	33.5	13.1	33.8	154.8	2.0
	CDC Bethune	12.7	36.6	47.6	49.0	13.8	49.1	208.8	2.3
101395	TMP-13195	12.9	41.7	37.6	33.6	14.9	35.0	175.7	1.5
101454	TMP-10109	9.2	28.6	30.6	37.6	9.6	33.8	149.5	2.2
97980	TMP-2412	9.0	26.4	28.1	29.0	10.5	30.0	133.0	1.9
101417	TMP-1920	15.0	43.7	37.0	39.1	17.5	35.6	187.9	1.5
	CDC Bethune	12.7	41.9	42.7	44.4	15.0	45.0	201.7	1.9
98398	TMP-2845	no seed material							
97639	TMP-7684	9.2	29.5	36.7	40.6	12.4	37.8	166.3	2.2
	Macbeth	14.6	43.3	45.0	60.1	18.0	50.7	231.7	2.1
	Hanley	12.9	39.3	38.7	47.6	16.3	42.6	197.4	1.9
	CDC Bethune	12.8	40.9	43.7	44.4	20.0	41.2	203.0	1.8
18991	TMP-1160	5.3	16.3	20.2	19.7	6.5	20.9	88.8	2.2
97147	TMP-8570	7.8	22.8	36.9	37.8	15.5	38.5	159.3	2.5
97306	TMP-2938	6.3	17.4	37.3	39.9	7.6	40.1	148.7	3.7
101580	TMP-10121	8.6	24.7	50.4	55.3	11.2	49.7	199.8	3.5
	CDC Bethune	12.9	39.5	44.1	55.8	16.9	42.2	211.4	2.1
101451	TMP-10033	9.5	33.9	36.8	37.8	10.7	32.2	160.9	2.0
97452	TMP-7529	12.5	38.2	32.5	34.7	16.0	32.1	166.0	1.5
100770	TMP-11317	13.0	37.1	46.6	47.0	14.2	47.0	205.0	2.2
101053	TMP-1792	10.1	26.2	25.6	27.5	10.6	29.1	129.0	1.8
	CDC Bethune	13.0	37.1	43.1	47.4	14.6	46.4	201.5	2.1
97334	TMP-8148	11.6	27.4	36.7	37.2	44.6	40.2	197.6	1.4
98275	TMP-2535	10.1	32.8	38.9	38.3	14.4	38.3	172.7	2.0
100799	TMP-8366	7.5	22.5	39.5	41.0	26.1	38.1	174.8	2.1
101565	TMP-10004	9.2	26.1	31.0	37.7	10.9	36.4	151.2	2.3
	CDC Bethune	13.0	40.3	44.4	49.2	16.4	46.1	209.4	2.0
97689	TMP-7723	11.0	27.6	41.1	44.6	10.5	46.5	181.2	2.7
18980	TMP-1070	no seed material							
97307	TMP-2939	8.3	17.4	35.9	22.6	6.4	35.6	126.2	2.9
	CDC Mons	15.1	36.8	47.1	52.3	16.8	49.7	217.9	2.2
	CDC Bethune	13.2	33.6	39.4	45.2	14.4	40.2	186.0	2.0
98057	TMP-2655	12.6	39.9	39.2	37.2	14.0	36.4	179.4	1.7
101594	TMP-10125	14.8	44.9	40.1	42.9	17.0	38.7	198.4	1.6
18979	TMP-1069	14.3	48.7	42.4	42.0	20.9	42.7	211.0	1.5
97610	TMP-2170	12.3	42.4	39.2	37.0	20.9	34.8	186.5	1.5
	CDC Bethune	13.2	34.9	43.2	45.6	15.0	41.2	193.1	2.1
33388	PGR-5039	12.7	32.2	37.1	40.5	14.3	35.3	172.1	1.9

101610	TMP-13685	9.1	28.1	30.3	33.3	10.8	31.2	142.6	2.0
98704	TMP-8230	7.1	25.5	37.6	37.7	18.1	37.1	163.0	2.2
96962	TMP-8451	8.2	21.5	40.4	40.4	11.1	41.8	163.5	3.0
	CDC Bethune	13.2	37.6	45.9	48.5	14.0	45.8	205.0	2.2
97670	TMP 7706-13	10.4	32.8	28.6	28.6	12.4	29.9	142.8	1.6
97953	TMP-2386				no seed material				
97083	TMP 2724-10	10.7	33.2	36.8	36.3	13.2	34.6	164.8	1.9
19158	TMP-1508	10.8	34.0	42.3	42.5	11.7	43.5	184.8	2.3
	CDC Bethune	13.2	35.1	38.5	45.8	14.6	40.7	188.0	2.0
101099	TMP-1838	9.9	29.1	31.1	31.9	13.1	33.8	148.9	1.9
98826	TMP-7516	10.4	31.4	36.0	38.2	13.0	37.9	166.9	2.1
98954	TMP-2261	12.7	39.7	33.0	33.3	14.9	34.0	167.7	1.5
	TMP-10097				no seed material				
	CDC Bethune	13.3	35.9	44.2	45.3	14.4	46.7	199.6	2.1
100797	TMP 8360-6	10.1	33.5	47.2	54.8	11.7	46.1	203.3	2.7
	TMP-2940				no seed material				
33386	PGR-5037	11.3	28.4	33.4	38.8	12.5	32.5	156.9	2.0
	TMP-2677				no seed material				
	CDC Bethune	13.3	38.9	47.2	51.6	16.2	52.2	219.4	2.2
100863	TMP-1435	9.9	31.5	36.5	50.7	14.4	40.6	183.4	2.3
	TMP 14331	13.8	43.0	48.4	48.1	17.5	46.5	217.2	1.9
101096	TMP-1835	11.5	35.7	34.5	31.3	13.1	33.5	159.6	1.6
101471	TMP-9922	8.2	25.0	35.0	38.9	7.9	38.8	153.9	2.7
	CDC Bethune	13.3	40.7	41.4	48.3	16.4	46.7	206.8	1.9
30860	PGR-1725	12.4	28.7	36.3	42.1	11.9	36.8	168.1	2.2
101386	TMP-13186	9.3	25.5	40.0	41.5	69.2	44.4	229.9	1.2
98946	TMP-2242	11.8	33.3	36.9	39.0	11.8	39.7	172.5	2.0
	TMP-8020				no seed material				
	CDC Bethune	13.3	41.3	42.0	44.5	15.6	41.5	198.3	1.8
98634	TMP-8158	9.4	24.4	38.6	41.9	10.1	37.7	162.3	2.7
101338	TMP-13137	11.7	37.5	48.5	42.8	12.1	41.4	194.0	2.2
101241	TMP-10817	9.9	32.1	35.3	34.2	12.0	32.1	155.6	1.9
98752	TMP-8284	11.7	31.0	45.4	44.6	12.9	42.0	187.5	2.4
	CDC Bethune	13.4	34.4	41.1	44.6	13.1	43.8	190.5	2.1
97642	TMP-7687	12.4	40.1	37.6	42.1	14.7	40.3	187.2	1.8
100629	TMP-11172	5.7	18.6	37.7	41.3	9.2	37.1	149.6	3.5
97458	TMP-2322	10.1	31.4	37.9	38.2	12.8	35.8	166.2	2.1
98734	TMP-8268	11.2	32.9	37.1	33.7	11.6	35.1	161.6	1.9
	CDC Bethune	13.5	43.1	42.1	55.5	17.9	45.3	217.4	1.9
33390	PGR-5041	10.1	27.8	47.5	49.3	11.7	51.1	197.6	3.0
97453	TMP-7530	12.4	36.7	43.1	49.7	14.3	46.0	202.3	2.2
101486	TMP-9935	11.0	31.1	38.3	40.6	14.1	37.3	172.3	2.1
19005	TMP-1466	10.6	28.7	33.8	39.8	12.0	36.1	161.0	2.1

	CDC Bethune	13.5	44.7	50.9	52.5	17.7	48.3	227.7	2.0
18989	TMP-1158	10.8	29.1	44.9	49.3	12.3	44.0	190.4	2.6
98276	TMP-2536	13.0	36.2	39.9	41.6	13.1	42.2	185.9	2.0
98794	TMP-8329	13.3	35.5	42.2	44.8	16.6	39.9	192.5	1.9
101385	TMP-13185	11.4	38.1	44.3	43.7	13.7	43.9	195.1	2.1
	CDC Bethune	13.6	34.1	42.1	45.6	13.0	45.2	193.7	2.2
100807	TMP-1368	6.3	18.9	33.0	34.9	7.6	29.7	130.4	3.0
101598	TMP-10126	5.0	16.6	25.3	31.4	9.1	28.2	115.6	2.8
101325	TMP-13124	9.2	27.7	36.3	39.9	8.6	33.4	155.1	2.4
98984	TMP-8172	8.2	27.9	34.4	31.5	11.3	34.8	148.0	2.1
	CDC Bethune	13.7	41.3	46.1	49.7	19.4	45.5	215.8	1.9
32546	PGR-4048	11.3	32.0	34.2	35.0	14.5	33.1	160.0	1.8
101419	TMP-1922	10.1	30.7	34.4	34.3	12.4	36.2	158.2	2.0
98903	TMP 2202-8	10.5	30.1	42.8	45.1	14.0	41.7	184.3	2.4
101595	TMP-10020	9.6	20.3	39.6	37.6	30.8	38.2	176.0	1.9
	CDC Bethune	13.8	43.0	48.0	54.9	17.2	48.6	225.6	2.0
96845	TMP-8373	23.3	24.3	43.2	57.9	492.0	38.7	679.3	0.3
	TMP-9981				no seed material				
97407	TMP-2988	11.2	30.3	31.0	32.9	12.0	35.3	152.7	1.9
101511	TMP-9953	6.5	21.6	42.3	39.8	9.6	43.2	163.0	3.3
	CDC Bethune	13.8	42.0	43.6	52.5	17.1	43.3	212.3	1.9
101405	TMP-13212	10.1	32.0	25.7	30.5	11.8	29.1	139.2	1.6
97649	TMP-7694	10.5	31.0	30.1	33.5	10.1	32.7	148.0	1.9
97444	TMP-7521	12.7	38.4	38.6	43.2	16.1	39.8	188.8	1.8
101463	TMP-10098	8.3	25.2	26.5	59.9	8.3	31.8	160.0	2.8
	CDC Bethune	13.8	42.3	44.2	48.3	15.5	47.4	211.5	2.0
97639	TMP 7684-11	8.7	26.9	35.7	36.4	11.2	35.7	154.5	2.3
	TMP-2894				no seed material				
100828	TMP-1396	12.4	36.0	46.5	46.7	14.3	41.4	197.2	2.1
98869	TMP-8482	12.4	30.6	41.8	45.1	12.5	40.3	182.7	2.3
	CDC Bethune	13.8	43.4	46.5	48.1	17.1	45.4	214.5	1.9
97520	TMP-7590	11.7	36.7	47.1	46.1	14.6	46.5	202.8	2.2
	Macbeth	15.4	48.5	49.0	51.3	18.0	47.1	229.2	1.8
101367	TMP-13166	12.8	41.0	38.8	40.1	14.1	38.2	185.0	1.7
	Prairie Blue	11.3	34.5	30.8	39.9	15.1	31.7	163.3	1.7
	CDC Bethune	13.9	31.4	36.9	42.9	13.2	40.9	179.2	2.1
96992	TMP-2088	7.7	22.7	29.6	39.0	8.4	33.8	141.3	2.6
98541	TMP-2934	12.8	36.8	44.6	43.8	11.9	47.2	197.1	2.2
100864	TMP-1437	6.1	24.6	32.0	41.1	13.2	38.6	155.7	2.5
33400	PGR-5051	17.5	32.0	36.7	44.5	14.7	39.5	184.8	1.9
	CDC Bethune	13.9	40.8	39.8	45.5	17.0	42.5	199.4	1.8
97958	TMP-2390	8.6	28.1	23.7	26.5	10.3	26.1	123.3	1.6
97396	TMP-2978	8.6	24.1	31.1	37.3	13.9	33.7	148.7	2.2

33397	PGR-5048	17.4	25.0	43.8	51.7	238.6	45.7	422.2	0.5
97287	TMP-2610	12.2	30.5	32.3	36.3	15.8	32.7	159.8	1.7
	CDC Bethune	13.9	37.8	45.3	57.5	14.9	44.8	214.2	2.2
98279	TMP-10221	14.3	37.9	37.7	38.3	13.0	38.2	179.3	1.8
98807	TMP-8340	12.1	32.7	50.0	47.9	21.7	50.9	215.3	2.2
101286	TMP-10873	9.7	30.2	31.6	34.1	11.2	32.4	149.2	1.9
97890	TMP-8382	9.3	28.2	36.8	40.5	13.7	40.9	169.5	2.3
	CDC Bethune	14.0	38.4	43.5	46.1	15.1	42.6	199.6	2.0
98150	TMP-2192	10.5	32.2	16.9	39.5	13.4	15.6	128.1	1.3
101366	TMP-13165	13.5	36.1	46.8	45.7	13.9	45.8	201.9	2.2
97489	TMP-7559	11.8	35.7	39.6	41.8	14.7	40.6	184.2	2.0
97430	TMP-2998	7.1	23.2	31.5	33.0	24.6	30.1	149.4	1.7
	CDC Bethune	14.0	48.1	43.3	43.5	16.0	45.2	210.2	1.7
100827	TMP-1395	7.0	22.1	32.8	34.5	15.4	30.0	141.8	2.2
101572	TMP-10118	16.9	36.1	42.7	53.4	19.6	39.8	208.5	1.9
	TMP-1423				no seed material				
101016	TMP-1719	11.8	34.9	36.5	40.8	12.0	35.5	171.4	1.9
	CDC Bethune	14.1	45.6	42.4	48.4	17.7	45.8	214.0	1.8
	Shape	12.9	36.8	41.9	48.6	14.8	43.1	198.1	2.1
97366	TMP-8641	12.8	40.9	34.5	36.7	13.6	34.3	172.9	1.6
101421	TMP-1924	15.8	46.4	41.3	40.5	17.9	38.9	200.8	1.5
100838	TMP-1409	10.5	31.5	40.1	39.1	12.6	37.6	171.5	2.1
	CDC Bethune	14.1	45.7	44.2	45.9	16.8	44.5	211.2	1.8
97350	TMP-8235	10.0	30.7	36.3	37.4	14.8	35.5	164.6	2.0
100547	PGR-14271	11.5	36.5	38.9	41.1	14.2	42.0	184.2	2.0
	Prairie Thunder	15.2	37.0	55.9	61.6	15.4	57.3	242.5	2.6
97584	TMP-7646	11.7	38.3	31.7	36.9	14.8	35.0	168.3	1.6
	CDC Bethune	14.1	36.9	44.2	47.3	14.0	47.7	204.1	2.1
97728	TMP-7760	7.4	20.3	24.7	32.9	6.6	30.8	122.8	2.6
98193	TMP-2530				no seed material				
101137	TMP-8784				no seed material				
101397	TMP-13197	12.8	35.0	34.1	37.2	15.4	37.8	172.2	1.7
	CDC Bethune	14.1	45.8	40.6	42.2	16.4	42.9	201.9	1.6
97586	TMP-7648	9.2	27.3	38.8	42.9	11.0	41.9	171.2	2.6
101496	TMP-13674	9.4	28.1	31.5	37.7	11.7	35.0	153.5	2.1
97533	TMP 7619-2	13.6	43.8	45.9	45.9	21.4	47.8	218.3	1.8
97470	TMP-10230	14.6	40.9	34.0	36.1	15.0	35.5	176.1	1.5
	CDC Bethune	14.2	34.1	41.8	45.8	14.0	44.8	194.7	2.1
19004	TMP-1311	12.1	34.8	29.0	31.6	15.3	31.0	153.9	1.5
	TMP-2862				no seed material				
97671	TMP-2173	12.5	35.1	41.3	43.3	13.2	42.3	187.7	2.1
98176	TMP-8479	12.2	36.4	40.2	38.3	14.7	38.5	180.2	1.8

	CDC Bethune	14.4	37.3	44.9	49.2	14.4	45.8	206.0	2.1
33385	PGR-5036	13.3	39.4	37.3	40.0	16.1	36.1	182.3	1.6
101114	TMP-1853	15.6	44.7	43.2	41.8	15.1	43.1	203.5	1.7
98467	TMP-2923	7.5	23.9	32.2	31.9	10.2	30.3	135.9	2.3
98303	TMP-2275	12.9	41.1	40.6	41.5	15.1	37.9	189.0	1.7
	CDC Bethune	14.4	45.7	45.0	50.0	17.1	47.4	219.5	1.8
97092	TMP-8556	11.7	37.4	48.9	44.9	13.5	48.6	205.0	2.3
100881	TMP-1455	8.8	26.2	35.8	39.9	15.8	34.8	161.4	2.2
98056	TMP-2181	14.0	32.8	17.5	40.0	14.1	15.2	133.6	1.2
97072	TMP 2713-4	11.5	26.7	28.6	30.3	10.6	30.5	138.2	1.8
	CDC Bethune	14.4	43.8	44.5	49.3	17.2	44.5	213.8	1.8
	TMP-1595				no seed material				
40081	PGR-13075	11.6	34.9	43.0	40.9	14.1	41.4	185.9	2.1
19001	TMP-1171	12.8	39.8	46.3	51.2	17.1	24.3	191.5	1.7
97487	TMP-7557	11.4	34.9	36.5	44.8	10.5	37.8	176.0	2.1
	CDC Bethune	14.5	39.8	44.8	48.5	15.7	47.0	210.3	2.0
98708	TMP-8233	11.8	38.8	39.6	40.5	23.2	34.7	188.6	1.6
98192	TMP-8006	8.3	26.6	26.7	27.3	11.6	28.4	128.9	1.8
98542	TMP-2622	14.1	41.9	54.6	57.6	19.5	51.1	238.8	2.2
97377	TMP-2968	11.9	31.5	52.0	55.1	11.9	52.2	214.5	2.9
	CDC Bethune	14.5	47.6	43.2	44.5	17.1	42.0	208.8	1.6
101230	TMP-10803	10.1	26.6	31.8	34.1	11.1	35.4	149.0	2.1
19160	TMP-1384	7.9	24.6	32.8	39.7	12.3	38.1	155.4	2.5
98639	TMP-8163	10.8	28.1	40.6	41.9	13.7	39.9	174.9	2.3
100883	TMP-1457	6.9	22.6	34.4	37.0	17.9	33.3	152.1	2.2
	CDC Bethune	14.5	43.8	48.1	54.7	17.9	49.3	228.2	2.0
97056	TMP-2697	12.1	31.2	45.2	43.7	10.9	48.1	191.1	2.5
101413	TMP-1174	12.9	38.6	33.8	39.0	14.4	37.4	176.1	1.7
98806	TMP-8339	10.5	23.1	28.6	33.0	8.4	30.4	133.9	2.2
101332	TMP-13131	10.3	32.3	39.2	43.7	13.6	41.0	180.0	2.2
	CDC Bethune	14.5	42.5	46.8	49.8	8.5	46.2	208.3	2.2
98733	TMP 8267-8	11.6	31.8	38.5	40.5	12.1	38.0	172.5	2.1
101039	TMP-1778	11.2	34.4	33.0	34.1	13.0	36.1	161.8	1.8
101416	TMP-1919	12.8	35.2	36.4	41.7	50.9	40.0	217.0	1.2
97749	TMP-7780	14.8	30.1	43.4	50.9	12.0	45.1	196.4	2.4
	CDC Bethune	14.5	48.0	46.2	47.2	16.7	50.0	222.6	1.8
98397	TMP-2844	14.0	42.6	40.3	38.3	15.9	36.5	187.4	1.6
97718	TMP-7751	6.6	19.8	30.0	37.4	15.7	31.2	140.8	2.3
98753	TMP-8285				no seed material				
	TMP-2953				no seed material				
	CDC Bethune	14.7	44.0	41.1	143.2	15.5	46.3	304.8	3.1
97139	TMP-2772	13.9	48.8	51.2	49.1	17.3	40.9	221.1	1.8
97587	TMP-7649	9.4	31.9	34.2	32.5	11.6	32.2	151.8	1.9

33992	PGR-5772	11.4	37.4	38.9	41.9	12.7	39.2	181.3	2.0
97403	TMP-2984	12.0	37.1	38.9	40.4	20.4	39.3	188.1	1.7
	CDC Bethune	14.8	43.0	42.5	49.0	19.3	48.1	216.6	1.8
98505	TMP-8598	9.6	30.6	28.9	29.7	10.6	27.4	136.8	1.7
	CDC Sorrel	16.6	51.1	31.2	34.7	40.0	33.6	207.3	0.9
97430	TMP 2998-9	7.3	24.4	38.2	38.7	26.9	36.3	171.8	1.9
97881	TMP-7880	11.2	33.2	27.4	31.1	12.4	30.2	145.5	1.6
	CDC Bethune	14.9	36.2	43.8	45.9	14.6	46.0	201.5	2.1
101127	TMP-1866	10.7	31.0	32.4	35.0	12.4	32.4	153.9	1.8
19157	TMP-1436	13.9	41.8	32.3	38.5	15.6	31.9	173.9	1.4
97129	TMP 2124-4	11.3	28.9	46.0	43.7	9.7	48.2	187.8	2.8
98037	TMP 2467-8	9.3	26.3	29.9	30.9	11.4	31.7	139.7	2.0
	CDC Bethune	14.9	45.8	50.6	50.5	18.9	46.7	227.3	1.9
97529	TMP-7597	16.4	47.4	47.3	46.7	16.6	49.9	224.3	1.8
98854	TMP-10222				no seed material				
101379	TMP-13179	13.6	40.5	43.4	44.4	17.2	42.7	201.8	1.8
18994	TMP-1163	11.3	35.1	37.8	40.0	17.2	35.6	177.1	1.8
	CDC Bethune	15.1	42.0	43.8	46.3	16.2	47.7	211.1	1.9
98157	TMP-7973				no seed material				
97584	TMP 7646-7	9.6	28.8	31.3	31.0	11.6	30.6	143.0	1.9
101392	TMP-13192	13.7	40.1	33.0	18.2	24.4	19.9	149.4	0.9
97571	TMP-7636	10.4	30.8	45.4	46.7	13.6	43.1	190.0	2.5
	CDC Bethune	15.2	37.2	43.2	46.6	16.0	46.5	204.8	2.0
97050	TMP-2695	10.2	30.8	40.2	42.2	12.1	41.3	176.8	2.3
97064	TMP-2705	11.4	22.7	41.5	41.8	8.9	40.7	167.1	2.9
98475	TMP-2279	7.3	22.4	32.8	34.4	17.2	35.1	149.2	2.2
101154	TMP-9786	12.8	37.2	37.5	40.9	14.0	39.9	182.3	1.9
	CDC Bethune	15.2	50.6	43.0	46.4	20.7	41.1	217.1	1.5
97129	TMP-2124	11.6	24.0	40.3	42.6	15.7	37.6	171.8	2.3
100848	TMP-1419	9.9	31.3	38.9	45.9	14.1	42.4	182.5	2.3
	TMP-2960				no seed material				
100841	TMP-1412	13.0	33.2	38.3	41.9	11.1	41.2	178.8	2.1
	CDC Bethune	15.3	38.3	43.2	49.3	15.2	48.2	209.5	2.0
101466	TMP-10135	12.4	39.9	38.4	38.1	15.0	37.0	180.8	1.7
97153	TMP-8574	7.5	24.2	37.3	37.2	9.8	35.6	151.5	2.6
100785	TMP-11332	11.8	37.7	33.9	43.3	13.7	32.4	172.8	1.7
98689	TMP 8216-5	5.0	16.4	37.0	35.9	6.6	31.5	132.3	3.7
	CDC Bethune	15.3	46.5	44.7	47.0	102.5	48.1	304.2	0.9
98135	TMP-2671	11.0	32.6	40.8	38.6	11.8	38.1	172.9	2.1
97740	TMP-7771	8.7	28.8	35.4	39.0	13.4	37.4	162.8	2.2
98072	TMP-2187	9.8	31.0	31.5	34.8	12.6	35.5	155.2	1.9
97393	TMP-2629	6.3	21.5	30.2	34.9	8.3	32.8	134.1	2.7
	CDC Bethune	15.4	46.5	41.8	48.8	16.7	43.1	212.3	1.7

97484	TMP-7554	8.8	26.0	33.0	34.8	9.7	37.2	149.5	2.4
97341	TMP 8152-12	12.7	40.2	35.5	35.2	14.6	35.5	173.7	1.6
98027	TMP-2457	10.6	33.8	36.1	34.9	11.6	34.9	161.9	1.9
35791	PGR-8233	13.0	38.0	35.4	41.9	17.4	38.3	184.1	1.7
	CDC Bethune	15.4	44.8	48.5	56.1	17.1	52.1	233.9	2.0
	TMP-9982				no seed material				
98363	TMP-2810	5.6	17.7	17.8	23.6	8.5	20.3	93.5	1.9
101559	TMP-10091	11.7	34.9	32.3	34.9	13.4	35.9	163.1	1.7
33389	PGR-5040	12.0	34.6	33.7	35.9	12.1	36.4	164.7	1.8
	CDC Bethune	15.4	46.5	41.3	52.8	18.3	47.3	221.5	1.8
98263	TMP-2534	8.2	26.2	41.0	39.6	9.2	41.0	165.2	2.8
97312	TMP-2948	6.9	22.0	25.0	26.7	7.5	24.0	112.1	2.1
	TMP-2280				no seed material				
97214	TMP-2564	7.8	22.7	34.6	45.4	16.4	38.1	165.1	2.5
	CDC Bethune	15.5	36.9	45.2	49.7	14.9	48.0	210.3	2.1
101404	TMP-13211	10.2	30.5	30.6	31.6	11.6	32.3	146.8	1.8
98742	TMP 8274-8	12.3	33.0	34.9	39.0	11.5	33.4	164.0	1.9
98278	TMP-2538	13.0	41.2	45.0	37.4	13.0	39.9	189.6	1.8
98165	TMP-7982	9.9	25.7	47.5	27.1	13.0	45.8	169.0	2.5
	CDC Bethune	15.5	50.8	46.8	44.5	17.9	45.7	221.2	1.6
19003	TMP-1310	12.9	38.4	34.3	35.4	12.2	41.0	174.2	1.7
101396	TMP-13196	10.7	31.5	31.7	32.5	14.1	30.2	150.6	1.7
97633	TMP-7678	6.4	19.7	33.0	37.0	8.3	40.6	145.1	3.2
37286	PGR-10014	15.6	36.6	43.8	49.8	15.4	41.8	203.1	2.0
	CDC Bethune	15.6	52.4	57.4	51.1	18.7	47.6	242.7	1.8
	Prairie Grande	14.1	41.9	46.0	49.4	14.0	51.9	217.3	2.1
98821	TMP-8123	8.3	23.4	29.3	35.8	8.4	31.9	137.3	2.4
96958	TMP 2650-14				no seed material				
101482	TMP-10083	6.3	19.4	34.5	38.9	8.0	35.9	143.1	3.2
	CDC Bethune	15.6	51.1	49.0	48.4	18.6	46.4	229.1	1.7
101403	TMP-13210	10.0	30.7	28.2	37.3	12.9	31.9	151.1	1.8
18982	TMP-1151	13.0	33.3	45.1	46.3	13.2	47.8	198.7	2.3
98934	TMP-2230	14.0	44.5	32.8	39.3	18.4	33.6	182.6	1.4
98056	TMP 2181-15	13.7	43.5	8.2	39.1	15.8	8.1	128.4	0.8
	CDC Bethune	15.6	44.5	39.0	131.7	15.5	44.7	291.0	2.8
97967	TMP-2399	9.6	29.7	37.3	40.6	12.5	37.6	167.3	2.2
98982	TMP-2922	5.8	18.4	31.7	32.8	8.9	33.0	130.6	2.9
	Hanley	13.3	39.7	38.1	44.2	17.0	40.5	192.8	1.8
98239	TMP-8535	6.2	20.4	58.2	55.7	9.1	56.5	206.1	4.8
	CDC Bethune	15.7	48.5	42.0	46.3	16.9	44.6	213.9	1.6
	TMP-2895				no seed material				
100837	TMP-1408	15.7	53.8	53.5	55.0	11.0	50.4	239.4	2.0
96974	TMP 2652-15	10.4	42.7	47.6	45.6	17.5	42.8	206.8	1.9

101055	TMP-1794	10.3	31.5	29.8	32.6	13.5	29.9	147.6	1.7
	CDC Bethune	15.7	49.2	48.4	44.7	17.7	44.9	220.7	1.7
	TMP 17618	11.4	36.8	40.9	43.1	13.4	43.7	189.2	2.1
98037	TMP-2467	12.5	35.8	40.2	36.1	12.1	36.6	173.3	1.9
100929	TMP-1614	12.8	38.8	38.5	35.0	8.3	35.5	168.9	1.8
	TMP-13163				no seed material				
	CDC Bethune	15.9	52.3	48.1	46.6	19.4	45.0	227.3	1.6
98923	TMP 2222-4	12.8	40.6	28.8	29.4	15.4	26.1	153.2	1.2
101118	TMP-1857	4.9	13.5	14.8	16.0	5.3	14.3	68.8	1.9
101378	TMP-13178	11.8	33.0	40.7	45.0	13.2	43.2	187.0	2.2
	TMP 2679-15				no seed material				
	CDC Bethune	16.0	49.4	40.0	124.1	17.1	46.6	293.2	2.6
101472	TMP-10082	11.5	31.0	45.2	47.6	10.0	48.3	193.7	2.7
	Lightning	12.2	38.8	42.3	51.0	15.9	49.2	209.3	2.1
98644	TMP 8168-3	8.8	23.1	36.7	32.7	10.4	33.8	145.4	2.4
97873	TMP-10239	14.7	39.1	43.6	46.3	13.7	41.1	198.5	1.9
	CDC Bethune	16.1	45.7	40.4	41.8	15.3	42.7	202.1	1.6
101448	TMP-10030	11.6	36.9	33.5	38.2	14.2	33.9	168.2	1.7
18997	TMP-1167	10.7	28.8	36.4	33.3	11.5	34.5	155.3	2.0
97871	TMP 2177-7	9.4	30.1	28.6	31.1	11.4	30.4	141.0	1.8
18987	TMP-1156	11.8	35.9	42.6	45.2	15.1	45.8	196.3	2.1
	CDC Bethune	16.3	51.8	47.3	49.3	19.2	44.3	228.1	1.6
101301	TMP-10889	13.7	41.7	34.8	36.1	16.3	32.3	174.9	1.4
98613	TMP-2963	7.0	23.7	41.7	41.1	9.8	43.2	166.4	3.1
97604	TMP-8375	13.5	39.1	39.3	40.9	15.1	41.3	189.2	1.8
100928	TMP-1613	7.3	19.1	31.3	32.2	14.5	30.8	135.2	2.3
	CDC Bethune	16.3	43.9	48.4	48.8	15.7	48.1	221.2	1.9
97406	TMP 2987-14	13.2	40.7	35.1	35.9	14.3	34.3	173.5	1.5
100678	TMP-11224	13.3	40.8	42.9	42.6	15.8	42.7	198.0	1.8
100895	TMP-1580	12.5	28.4	38.2	41.0	13.5	35.7	169.4	2.1
101331	TMP-13130	9.8	29.4	39.3	40.5	13.3	42.5	174.8	2.3
	CDC Bethune	16.4	36.3	46.2	47.7	50.3	47.7	244.7	1.4
	Hanley	13.6	44.2	42.1	44.7	17.4	42.1	203.9	1.7
	Macbeth	16.6	52.5	51.7	52.5	19.9	51.1	244.3	1.7
	TMP-2795				no seed material				
98109	TMP-2659	9.8	32.8	31.0	31.9	14.4	29.5	149.4	1.6
	CDC Bethune	16.6	53.6	47.7	47.2	18.8	44.2	228.1	1.6
101265	TMP-10847	7.4	25.1	29.7	29.0	10.2	28.5	130.1	2.0
98812	TMP-8345	10.2	25.9	31.2	38.7	11.3	34.8	152.1	2.2
	TMP-8017				no seed material				
100885	TMP-1460				no seed material				
	CDC Bethune	16.9	51.9	48.0	48.7	18.1	47.1	230.7	1.7
	TMP-10021				no seed material				

97321	TMP-8126	7.5	24.2	30.9	34.0	10.1	31.0	137.8	2.3
	TMP-9984				no seed material				
101136	TMP-1876	13.4	40.6	40.3	41.5	16.2	40.2	192.1	1.7
	CDC Bethune	17.1	39.6	46.9	52.8	15.4	47.8	219.7	2.0
52732	PGR-27314	15.1	45.8	42.6	43.5	17.1	40.2	204.2	1.6
97907	TMP-7899	9.5	28.5	42.6	50.9	11.1	47.8	190.3	2.9
101208	TMP-9853	9.0	26.9	33.3	36.0	9.8	32.6	147.6	2.2
101116	TMP-1855	10.4	32.1	32.1	32.6	13.3	33.6	154.1	1.8
	CDC Bethune	17.5	53.5	50.0	48.8	19.1	46.8	235.7	1.6
98286	TMP-2269	13.2	41.3	39.3	39.7	16.7	37.5	187.7	1.6
97961	TMP-2393	11.6	36.6	33.8	34.6	13.9	31.8	162.3	1.6
101237	TMP-10812	12.9	42.9	48.0	45.0	16.5	42.5	207.8	1.9
101402	TMP-13203	11.1	34.4	38.4	39.8	13.9	34.9	172.5	1.9
	CDC Bethune	17.6	51.4	43.0	43.7	18.3	44.0	218.0	1.5
98263	TMP 2534-5	9.4	26.9	49.8	46.8	9.5	47.9	190.1	3.2
97096	TMP-2756	13.6	37.8	47.9	49.9	15.0	46.9	211.1	2.2
97300	TMP-8073	11.8	38.8	33.6	31.1	14.2	31.7	161.2	1.5
100851	TMP-1422	8.5	24.3	43.7	52.1	24.9	47.1	200.6	2.5
	CDC Bethune	17.9	41.0	47.0	50.4	16.8	48.6	221.8	1.9
32542	PGR-4044	21.1	32.6	37.6	44.1	66.4	34.2	236.0	1.0
	TMP-2924				no seed material				
33393	PGR-5044	14.7	41.7	36.0	36.3	13.9	35.0	177.6	1.5
100939	TMP-1507	9.3	27.8	40.4	42.5	12.1	40.6	172.6	2.5
	CDC Bethune	18.2	35.6	45.9	47.4	97.6	44.4	289.1	0.9
97238	TMP-2932	10.4	31.5	37.3	36.5	15.5	38.9	170.1	2.0
101296	TMP-10884	10.0	27.1	32.3	30.8	11.1	34.9	146.3	2.0
97503	TMP 7573-6	11.3	34.4	40.9	43.6	16.3	43.2	189.7	2.1
98100	TMP-2508	8.2	24.3	42.7	44.7	26.5	43.6	190.0	2.2
	CDC Bethune	18.3	42.0	46.1	48.4	15.4	46.2	216.4	1.9
	Macbeth	18.0	41.7	46.5	55.7	53.0	46.8	261.8	1.3
	Hanley	14.2	42.5	48.0	44.4	18.5	42.7	210.3	1.8
	TMP-2817				no seed material				
101299	TMP-10887	13.0	39.3	40.0	40.7	14.6	39.5	187.1	1.8
	CDC Bethune	18.4	39.0	48.2	28.7	46.2	45.2	225.7	1.2
101375	TMP-13175	12.4	41.1	39.0	37.2	14.7	38.3	182.7	1.7
19017	TMP-8663				no seed material				
	TMP-13194				no seed material				
97616	TMP-7670	11.4	34.6	33.2	34.5	15.6	31.7	161.0	1.6
	CDC Bethune	18.6	40.9	45.8	51.0	15.5	47.0	218.8	1.9
97424	TMP-2147	10.7	32.7	34.1	39.0	11.5	38.8	166.8	2.0
	TMP-2160				no seed material				
101279	TMP-10862	15.1	32.3	41.5	46.8	11.3	44.5	191.6	2.3
101308	TMP-13107	13.4	36.5	48.6	51.6	13.9	48.3	212.3	2.3

	CDC Bethune	19.3	46.8	48.2	32.2	17.5	45.6	209.5	1.5
98364	TMP-2811	10.9	34.0	34.4	35.1	13.5	35.2	163.1	1.8
101240	TMP-10816	13.1	40.0	34.0	36.7	15.5	32.6	172.0	1.5
101327	TMP-13126	7.1	31.6	32.3	31.4	10.6	32.9	145.9	2.0
18981	TMP-1097				no seed material				
	CDC Bethune	20.5	36.9	40.1	51.8	10.1	46.6	205.9	2.1
101132	TMP-1871	14.8	26.7	37.3	46.5	84.3	41.7	251.4	1.0
98535	TMP-8643				no seed material				
18986	TMP-1155	11.5	35.1	34.5	38.9	15.5	17.9	153.4	1.5
101600	TMP-10024	10.4	30.5	29.2	34.2	10.0	42.3	156.5	2.1
	CDC Bethune	20.5	39.2	42.1	50.1	16.9	44.8	213.6	1.8
101401	TMP-13202	11.2	33.9	37.1	36.9	14.3	36.9	170.4	1.9
97004	TMP-8464	6.0	19.2	32.4	38.5	7.8	30.9	134.7	3.1
97679	TMP 7714-14	9.5	26.8	39.6	45.0	13.1	38.3	172.4	2.5
	TMP 2679-9				no seed material				
	CDC Bethune	21.8	38.1	46.9	34.4	78.5	43.3	263.1	0.9
101119	TMP-1858	14.5	40.7	35.8	39.2	17.5	36.4	184.1	1.5
97679	TMP-7714	12.8	35.1	41.9	49.4	12.9	42.8	194.8	2.2
101310	TMP-13109	8.9	26.5	40.4	45.5	20.9	38.8	181.0	2.2
101026	TMP-1729	7.9	25.3	36.5	36.3	11.7	36.8	154.4	2.4
	CDC Bethune	23.4	44.3	50.3	55.7	77.5	46.4	297.6	1.0
96988	TMP-2084	8.9	29.0	48.2	48.0	12.6	42.2	188.9	2.7
100797	TMP 8360-8	12.4	39.7	51.5	55.7	15.4	49.0	223.7	2.3
18998	TMP-1168	11.5	36.5	41.3	45.3	15.7	42.6	192.9	2.0
18988	TMP-1157	11.1	28.2	41.8	44.8	12.5	40.7	179.2	2.5
	CDC Bethune	24.3	45.5	50.3	57.3	106.3	47.7	331.4	0.9
98254	TMP-8018	7.7	27.2	37.0	33.4	10.2	34.0	149.4	2.3
18973	TMP-605	16.0	47.3	53.6	54.3	15.5	56.9	243.6	2.1
97392	TMP-2975				no seed material				
101348	TMP-13147	15.0	48.0	48.2	47.9	17.3	48.8	225.3	1.8
	CDC Bethune	24.7	42.6	20.3	46.5	15.2	41.2	190.4	1.3
100674	TMP-11220	12.8	24.2	35.1	43.9	11.2	37.5	164.8	2.4
	TMP-9941				no seed material				

Data are presented as mean value from two replications

APPENDIX E

PROFILE OF CYCLOLINOPEPTIDES IN ACCESSIONS 'HOLLANDIA' AND 'Z 11637'OF CORE COLLECTION

Table E.1 Profile of CLs (mAU) in oxidized methanol extract of 'Hollandia' flax

Location	Year	1-Mso,3-Mso-CLF	1-Mso,3-Mso-CLG	1-Mso-CLB	3.10	1-Mso-CLE	1-Mso-CLD	CLA	Total	Ratio
Saskatoon, SK	2009	18.3	59.3	0.0	44.5	65.5	22.1	0.0	165.2	0.7
	2010	12.8	36.7	0.0	33.1	37.3	14.6	0.0	100.7	0.6
	2011	14.2	40.1	0.0	29.6	37.6	15.1	0.0	107.0	0.5
Morden, MB	2009	15.2	42.6	0.0	32.7	36.0	16.2	0.0	110.0	0.5
	2010	13.9	40.6	0.0	26.7	39.4	17.6	0.0	112.3	0.5
	2011	15.4	43.4	0.0	30.4	39.2	19.2	0.0	116.5	0.5

Data are presented as mean value from two replications

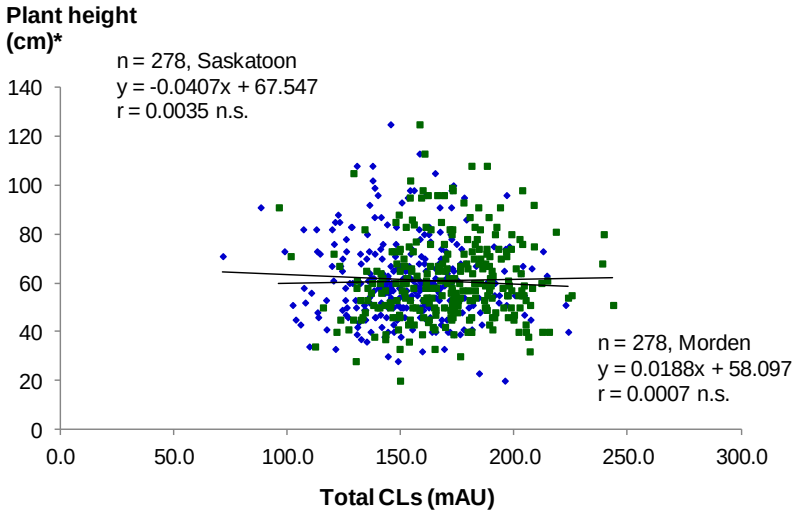
Table E.2 Profile of CLs (mAU) in oxidized methanol extract of 'Z 11637' flax

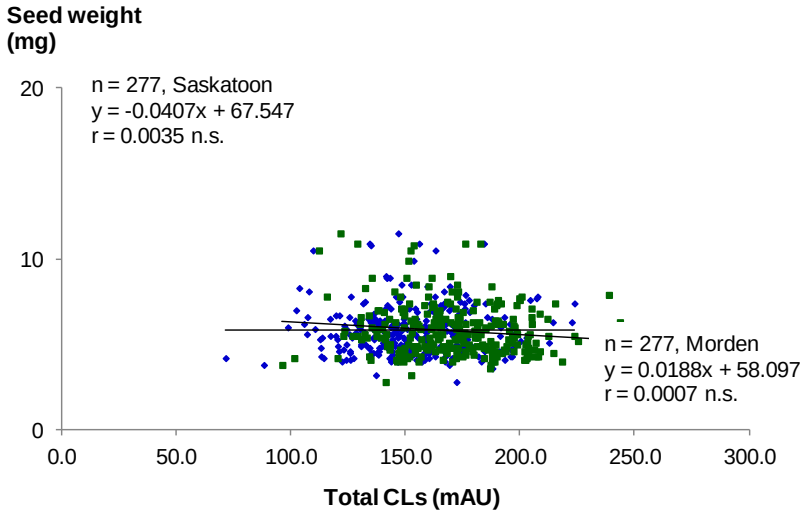
Location	Year	1-Mso,3-Mso-CLF	1-Mso,3-Mso-CLG	1-Mso-CLB	3.10	1-Mso-CLE	1-Mso-CLD	CLA	Total	Ratio
Saskatoon, SK	2009	13.3	42.9	0.0	36.6	36.4	14.6	0.0	107.3	0.5
	2010	6.5	20.8	0.0	23.9	27.6	8.1	0.0	63.0	0.8
	2011	14.4	45.3	0.0	31.5	34.7	16.9	0.0	111.3	0.5
Morden, MB	2009	14.1	40.8	0.0	34.4	35.6	14.5	0.0	105.0	0.5
	2010	14.7	45.8	0.0	35.1	40.3	17.5	0.0	118.4	0.5
	2011	13.2	44.2	0.0	33.5	36.2	17.4	0.0	111.0	0.5

Data are presented as mean value from two replications

APPENDIX F

CORRELATION OF CYCLOLINOPEPTIDE CONTENT TO QUANTITATIVE PLANT MORPHOLOGICAL TRAITS





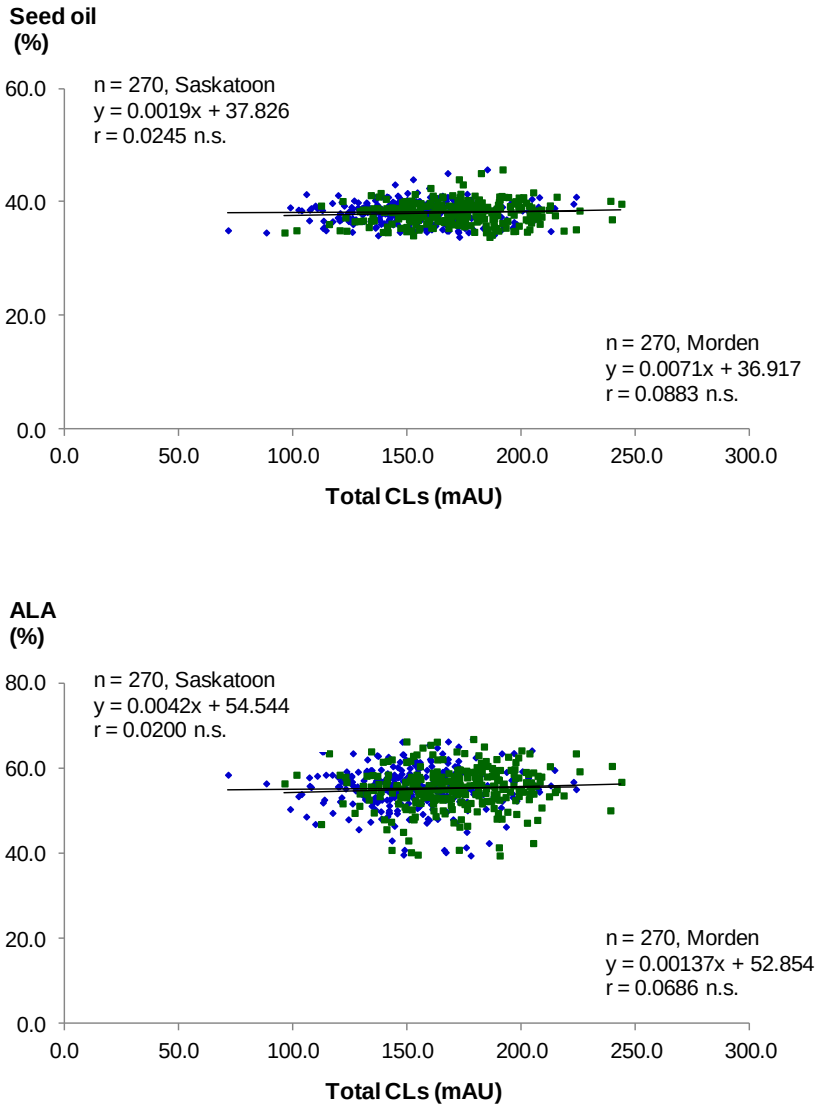


Figure E. Correlation between plant height, seed weight, total seed oil content, and ALA content to the total level of cyclolinopeptides expressed by the core collection planted in Saskatoon, SK and Morden, MB in 2009. n.s.: not significant

APPENDIX G

CORRELATION OF CYCLOLINOPEPTIDE CONTENT TO PLANT QUALITATIVE MORPHOLOGICAL TRAITS

Table G. Total level of cyclolinopeptides expressed by the core collection planted in Saskatoon, SK and Morden, MB in 2009 according to plant qualitative traits.

Qualitative traits			SK					MB				
		n	Mean	Min	Max	SD	CV	Mean	Min	Max	SD	CV
Seed colour	light brown	3	135.4	122.2	148.6	18.7	13.8	171.0	149.0	191.5	21.2	12.4
	medium brown	223	152.9	71.4	222.6	24.4	16.0	169.9	96.2	243.6	24.6	14.5
	mottled	9	154.4	112.6	184.2	23.4	15.1	168.0	122.8	197.2	22.1	13.2
	olive	8	164.2	128.0	223.7	30.5	18.6	182.8	152.1	215.3	20.6	11.3
	yellow	24	154.5	113.1	207.6	25.2	16.3	169.1	115.6	208.5	26.4	15.6
Stem branching	1/1 branched	7	162.6	145.6	183.0	13.6	8.4	174.1	148.0	197.2	17.9	10.3
	1/2 branched	58	153.0	107.1	204.3	24.5	16.0	167.9	112.1	223.7	28.1	16.7
	1/3 branched	101	154.4	103.6	223.7	23.2	15.0	170.5	132.3	218.3	20.9	12.2
	1/4 branched	63	152.1	88.1	222.6	25.2	16.6	172.7	96.2	243.6	23.2	13.4
	1/5 branched	38	155.1	71.4	212.6	28.1	18.1	175.7	101.4	239.4	31.1	17.7
	1/6 branched	11	139.3	120.2	172.6	18.1	13.0	157.6	129.0	187.9	21.1	13.4
Petal colour	blue	101	152.8	98.6	222.6	26.1	17.1	171.5	112.1	243.6	25.2	14.7
	dark blue	1	154.2	154.2	154.2	_	_	205.0	205.0	205.0	_	_
	light blue	3	168.4	167.5	169.3	1.3	0.7	181.9	176.1	187.5	5.7	3.1
	mixture	3	151.1	141.4	160.8	13.7	9.1	167.4	163.5	169.5	3.4	2.1
	pink	9	163.1	126.1	204.3	27.1	16.6	174.5	115.6	223.7	31.8	18.2
	lavender	73	148.0	71.4	207.6	24.1	16.3	168.4	101.4	239.4	26.2	15.5
	violet	35	154.2	122.2	195.8	19.8	12.8	172.0	130.1	212.3	21.9	12.7
	white	51	157.2	88.1	223.7	25.3	16.1	170.9	96.2	238.8	23.6	13.8